CHARACTERIZATION OF C_4 PHOTOSYNTHESIS IN SODIUM-DEFICIENT PLANTS

by

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STATEMENT

The work presented in this thesis is my own. Specific contributions and co-operative work with others are referred to in the text.

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To Helen, Jeremy and my mother, thanks. (and Suellen too).

How far I promise not; the way is long and dark; and as Travellers sometimes among Mountains, by gaining the top of one, are so far from their journeys end; that they only come to see that another lies before them; so the way of Nature is, so impervious and as I may say, Down Hill and Up Hill, that how far soever we go, yet the surmounting of one difficulty, is want to give us prospect of another.

NEHEMIAH GREW (1682)
Anatomy of Plants

ABSTRACT

This thesis examines various aspects of the photosynthetic carbon metabolism of sodium-deficient and control C_4 plants. In representative species from each of the C_4 decarboxylation sub-groups, sodium-deficient plants retain all of the characteristic features of C_4 photosynthetic metabolism. The leaf anatomy and ultrastructure, plant carbon isotope composition ($\delta^{13}C$), CO_2 compensation point and radiotracer kinetic characteristics during steady-state photosynthesis in sodium-deficient plants show that, although the rate of photosynthesis is reduced, the C_4 pathway does remain functional in these plants.

Two specific hypotheses concerning the essential role of sodium in C_4 photosynthesis were examined. These are;

(*a*) that sodium is required for the formation or activation of those enzymes involved in the conversion of pyruvate to phosphoenolpyruvate during C_4 photosynthesis,

and, (*b*) that the bundle-sheath compartment of sodium-deficient C_4 plants is "leakier", resulting in a reduction in photosynthetic efficiency due to either increased back-diffusion of CO_2 (released during decarboxylation of C_4 acids), or enhanced RuP_2-oxygenase activity.

In response to the first hypothesis, *in vitro* activities of pyruvate, Pi dikinase, and a number of other enzymes involved in C_4 photosynthesis were examined. Sodium-deficient C_4 plants possessed high activities of pyruvate, Pi dikinase and the capacities for substrate-dependent oxygen evolution by intact mesophyll chloroplasts, provided strong evidence that the conversion of pyruvate to phosphoenolpyruvate in these organelles was unimpaired. In addition, the kinetics of ^{14}C-labelling during steady-state photosynthesis in sodium-deficient leaves did not provide conclusive evidence of a single metabolic block in the conversion of pyruvate to phosphoenolpyruvate.

To examine the second hypothesis, that sodium-deficient leaves are "leakier", physiological properties of whole leaf gas exchange in sodium-deficient plants were characterized. Responses of photosynthetic CO_2 assimilation to intercellular $p(CO_2)$, irradiance, atmospheric oxygen concentration, and the components of photosynthetic oxygen exchange in sodium-deficient and control leaves were compared. As photosynthesis in sodium-deficient plants was unaffected by changing oxygen concentrations, and quantum yields of photosynthetic CO_2 assimilation were similar in sodium-deficient and control leaves it seems unlikely that the bundle-sheath compartment of sodium-deficient plants is "leakier".

Two specific characteristics of sodium-deficient C_4 plants which may be pertinent to the overall lower level of photosynthetic activity were noted. First, radiotracer kinetic studies showed that sodium-deficient plants have increased labelling of the free protein amino acids alanine, glycine and serine. This was consistent with the presence of much larger pool sizes of these metabolites in the leaves of sodium-deficient plants. Second, the intercellular $p(CO_2)$, obtained at ambient CO_2 concentrations in sodium-deficient leaves were consistently higher than those of controls. This characteristic was strong evidence that stomatal conductance does not limit photosynthetic capacity in sodium-deficient plants.

Sodium-deficient plants were further characterised by earlier saturation of the responses of photosynthetic CO_2 assimilation to both irradiance, and intercellular $p(CO_2)$. In addition, the relative activities of certain mesophyll and bundle sheath enzymes in sodium-deficient and control plants were different. From these observations it was concluded that disproportionate activities of carbon metabolism and photophosphorylation on the one hand, and mesophyll and bundle sheath metabolism on the other, might underlie C_4 photosynthesis in sodium-deficient plants.

ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumen
CAM	Crassulacean acid metabolism
DTT	Dithiotreitol
EDTA	Ethylenediamine tetraacetate
HEPES	(N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid)
K_m	Michaelis constant
(+Na)	Control plant (received 0.1 mol m^{-3} NaCl(2.3ppm sodium))
(-Na)	Sodium-deficient plant (no sodium added)
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
OAA	Oxaloacetate
$p(CO_2)$	partial pressure of CO_2
PCR	Photosynthetic carbon reduction cycle
PEP	Phosphoenolpyruvate
Pi	Orthophosphate
RuP_2	Ribulose 1,5-biphosphate

MAJOR SYMBOLS (UNITS)

A (μmol $m^{-2}s^{-1}$)	rate of CO_2 assimilation
g (mol $m^{-2}s^{-1}$)	total leaf conductance to water vapour transfer
I (Einstein $m^{-2}s^{-1}$)	irradiance
P_a (μbar)	external partial pressure of CO_2
P_i (μbar)	intercellular partial pressure of CO_2

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CHAPTER 1

INTRODUCTION

When sodium is supplied at relatively high concentrations, ie. often at 10 mol m^{-3} (230 ppm) and above, it increases the dry weight yield or induces qualitative changes in certain higher plants (for reviews, see Marschner 1975, Flowers *et al.* 1977, Jennings 1976, Brownell 1979). However when sodium is supplied at low concentrations, ie. 0.1 mol m^{-3} (2.3 ppm) and below, it has been found to be an essential micronutrient element only for those higher plants possessing the C_4 dicarboxylic pathway of photosynthesis (Brownell and Crossland 1972). A comprehensive review of sodium as an essential micronutrient element for plants and its possible role in metabolism was recently presented by Brownell (1979).

The experiments in this thesis are solely concerned with the sodium requirement of C_4 plants. In this chapter I will briefly outline the responses of various higher plants to sodium at low concentrations, ie. less than 0.1 mol m^{-3} (2.3 ppm). Known metabolic and physiological effects of sodium in these plants are then examined, and our current understanding of its possible role as an essential micronutrient element is discussed.

1.1 SODIUM - AN ESSENTIAL MICRONUTRIENT ELEMENT FOR SOME HIGHER PLANTS

Studying the growth of *Atriplex vesicaria* Heward under conditions where sodium was rigorously excluded, Brownell and Wood (1957) were the first to demonstrate a specific requirement for sodium in a higher plant. In a subsequent study (Brownell 1965) sodium was shown to be an essential micronutrient element for *A. vesicaria* according to the rigidly defined criteria of essentiality proposed by Arnon and Stout (1939). Plants of *A. vesicaria* grown in "sodium free" cultures, ie. containing less than 0.07 mmol m^{-3} (0.0016 ppm) sodium as an impurity, showed characteristic deficiency symptoms, with yellowing of their leaves and development of white necrotic patches on leaf tips and margins. In some cases plants died at an early stage thereby satisfying the first of the criteria of Arnon and Stout (1939), viz. "a deficiency of it makes it impossible for the plant to complete the vegetative or reproductive stage of its life cycle." Of the group I elements - lithium, sodium, potassium and rubidium - only sodium brought about the recovery of sodium-deficient plants, therefore satisfying the second of the criteria, viz. "such deficiency is specific to the element in question, and can be prevented or corrected only by supplying this element."

The third of the criteria, viz. "the element is directly involved in the nutrition of the plant quite apart from its possible effects in correcting some unfavourable microbiological or chemical condition of the soil or other culture medium", could not be wholly fulfilled by Brownell (1965) as a specific essential role for sodium in the metabolism of the plant was not determined at that time. However, foliar applications of sodium can bring about either localized or complete recovery in sodium-deficient plants depending upon the amount

of sodium supplied (Mill 1977, Brownell 1979). These results suggest a direct effect of sodium on the plant as distinguished from its possible effect on the root environment.

This work was extended by Brownell (1968) in order to establish whether this requirement for sodium was general for higher plants. The results of these experiments suggested that sodium was apparently essential for an extremely restricted group of higher plants. While Australian species of *Atriplex* showed three- to thirty-fold differences in yield response to 0.1 mol m^{-3} (2.3 ppm) sodium, and exhibited characteristic symptoms of sodium-deficiency, some other species of *Atriplex*, sugar beet, barley, several legumes, lettuce, tomato and *Aster tripolium*, were largely indifferent to this sodium treatment. Although barley (*Hordeum vulgare* L.c.v. Pallidium) and *Atriplex hortensis* L. var. *astrosanguineae* (Brownell 1968), and tomato (*Lycopersicum esculentum* Mill. c.v. Grosse Lisse)(Woolley 1957) have shown marginal yield responses to low concentrations of sodium, there were no visible symptoms of sodium-deficiency in these species.

Following elucidation of the C_4 dicarboxylic photosynthetic pathway (Hatch and Slack 1966, 1970) it was evident that those species of *Atriplex* which required sodium also possessed characteristics of the C_4 photosynthetic pathway. Subsequently, Brownell and Crossland (1972) established the correlation between the essentiality of sodium and the possession the C_4 photosynthetic pathway. This correlation thus effectively fulfills the third criterion of Arnon and Stout (1939). The work described in this thesis goes somewhat further in examination of the mechanisms involved.

To date, including results presented in a following section (Chapter 2, Section 2.3.2), a requirement for sodium has been demonstrated in a wide range of C_4 plants, which includes certain

species of *Cynodon, Chloris, Digitaria, Echinochloa, Eleusine, Panicum* (Poaceae), *Kyllinga* (Cyperaceae), *Atriplex, Halogeton, Kochia* (Chenopodiaceae), *Amaranthus* (Amaranthaceae) and *Portulaca* (Portulacaceae). Within two genera, *Atriplex* and *Kochia,* which include both C_3 and C_4 species, only the C_4 species responded to sodium by marked increases in dry weight (Brownell and Crossland 1972). Furthermore, those C_4 species which have been shown to require sodium include representative species from each of the C_4 acid decarboxylation sub-groups defined by Hatch *et al.* (1975). Contrary to recent suggestions of Clarkson and Hanson (1980), there is no direct correlation between the requirement for sodium as a micronutrient and the possession of halophytic or sodium-accumulating properties (Brownell 1979).

Responses to sodium at low levels have also been obtained in the crassulacean acid metabolism species *Bryophyllum tubiflorum* (Brownell and Crossland 1974, Boag 1976). In this species significant yield responses to 0.1 mol m^{-3} sodium are only obtained when plants are grown under conditions of short day length and large diurnal temperature variation. Under this growth regime a large proportion of total carbon assimilate is obtained by nocturnal CO_2 fixation *via* phosphoenolpyruvate carboxylase (Brownell and Crossland 1974, Boag 1976).

It would seem that sodium might be considered to be a unique essential element; it is only essential for C_4, and perhaps CAM species, but not for C_3 species of higher plants. However, the possibility that sodium is essential in minute amounts for C_3 species should not be dismissed. In discussing essential and functional aspects of trace elements Hewitt (1979) suggests that, "in all cases of negative evidence for a requirement there remains the possibility, which cannot be eliminated under practical limits of experimentation, that requirements

are positive but not detectable. These limits are in the region of 10^3 atoms/cell or 10^{-5} μM." In the experiments of Woolley (1957) the concentration of sodium in the shoots of tomato, a C_3 species, grown in a "sodium free" culture solution, ie. containing less than 0.35 mmol m^{-3} sodium as an impurity, was 89 μmol kg^{-1} dry weight (0.25 ppm) which is orders of magnitude in excess of the lower limit suggested by Hewitt (1979). What can be stated definitely is that C_4 plants, and the CAM species *B. tubiflorum*, require a sodium concentration of about 0.1 mol m^{-3} (2.3 ppm) for optimal growth, and that this is approximately 1250 times the concentration of sodium remaining as an impurity in a basal culture solution which supports optimal growth of C_3 species (Brownell 1979).

In addition, small amounts of sodium are specifically required for the growth of certain blue-green algae. For example, Allen and Arnon (1955) showed a requirement for sodium as a micronutrient element in *Anabaena cylindrica* and determined that 0.2 mol m^{-3} sodium (5 ppm) was required for optimal growth in this species. Examining the effects of sodium on the nitrogen metabolism of *A. cylindrica*, Brownell and Nicholas (1967) found that higher levels of sodium were required to avoid deficiency symptoms in those cultures grown in nitrate (ie. 0.4 mol m^{-3} sodium) as compared to those cultures grown without combined nitrogen (ie. 0.04 mol m^{-3} sodium). The growth responses to added sodium obtained in other species of blue-green algae (Allen 1952, Kratz and Myers 1955, Bostwick *et al.* 1968) would suggest that sodium might be generally essential for members of Cyanophyta. Sodium is also essential, at concentrations generally in excess of 50 mol m^{-3}, for certain marine bacteria and fungi and certain non-halophytic bacteria (for review see Brownell 1979).

1.2 METABOLIC AND PHYSIOLOGICAL EFFECTS OF SODIUM IN C_4 AND CAM PLANTS

In attempting to determine a specific role for sodium in plants for which it is essential Brownell and Jackman (1966) investigated physiological changes following the supply of sodium to sodium-deficient plants of *Atriplex nummularia* and *Atriplex inflata*. During the recovery of *A. nummularia* there was no growth response until after the sixth day. However, the concentration of sodium in the leaves increased rapidly from the time of sodium application (ie. 0.6 mol m^{-3}) and by the fifth day all the sodium had been removed from the culture medium. Of the physiological parameters measured the total chlorophyll content of the leaves and the respiration rate per unit fresh weight of shoots responded most readily to sodium. In addition, the concentrations of sugars and starch increased and the ratios of soluble to total nitrogen decreased slowly during recovery from sodium deficiency. The respiratory response was further characterized by Brownell and Jackman (1966). This respiratory response was not due to removal of substrate limitation, it was specific to sodium and both growth and respiration exhibited similar relationships between response and concentration of sodium in the culture solution. As the rate of anaerobic CO_2 production increased when sodium was fed to leaves, Brownell and Jackman (1966) suggested that sodium acts in the glycolytic stages of respiration. Importantly, Mill (1977) did not observe similar increases in the respiratory rate of leaves when sodium was supplied to sodium-deficient plants of *Kochia childsii* and *Amaranthus tricolor*. These results imply that the effect of sodium in increasing the respiration rate of leaves is not general for all C_4 species and Brownell (1979) suggests that this response in *Atriplex* species might be related to their propensity for accumulating sodium from low substrate concentrations.

In recognizing the correlation between the possession of the C_4 photosynthetic pathway and the essentiality of sodium, Brownell and Crossland (1972) suggested that sodium would be expected to function in a metabolic system unique to C_4 plants. In addition, the responses to sodium obtained in the CAM plant *B. tubiflorum*, grown under conditions where plants would be more dependent upon dark fixation of CO_2 (Brownell and Crossland 1974), further implied that sodium might function within the C_4 dicarboxylic acid system operating in C_4 and CAM plants: but not in C_3 plants.

Both CAM and C_4 photosynthesis involve CO_2 assimilation *via* a preliminary carboxylation, catalysed by PEP carboxylase, and a subsequent decarboxylation of the C_4 acids *via* one of three decarboxylase systems involving NAD- or NADP-malic enzyme or PEP-carboxykinase. The CO_2 released is then refixed by RuP_2 carboxylase under conditions resulting in increased CO_2 concentrations in the vicinity of this enzyme (for reviews see Hatch and Osmond 1976, Hatch 1976a,b, Edwards and Huber 1981, Osmond 1978, Osmond and Holtum 1981). While there are numerous biochemical similarities between CAM and C_4 photosynthesis, these two processes are physiologically quite different. Essentially, dicarboxylic acid synthesis is temporally separated from decarboxylation and refixation of endogenously released CO_2 during CAM, and spatially separated during C_4 photosynthesis.

To investigate the hypothesis that sodium might be involved in the C_4 dicarboxylic acid system, Holtum (1975) and Brownell (1979) determined the activities of PEP carboxylase in sodium-deficient and control C_4 plants. They established that there was no substantial difference between the specific activity on a fresh weight or soluble protein basis of this enzyme extracted from either shoots, or roots, of sodium-deficient and normal plants. Furthermore, preliminary ^{14}C-labelling

experiments with sodium-deficient C_4 plants did not suggest a direct effect of sodium on the activity of PEP carboxylase *in vivo* (Holtum 1975, Webb 1977, Brownell and Osmond, unpublished results).

The effects of sodium nutrition on the C_4 dicarboxylic acid system of CAM plants were determined by following the diurnal patterns of carboxylation (ie. acidification) and decarboxylation (ie. deacidification) in *B. tubiflorum* grown under both long- and short-day regimes (Boag 1976). Under both growth regimes, observed rates of dark acidification, magnitudes of acid accumulation and rates of deacidification in the light were similar in sodium-deficient and control plants. Such results suggested that sodium did not have a direct effect upon either the process of acidification in the dark (ie. primary fixation via PEP carboxylase, reduction of OAA and transport of malic acid into the vacuole) or the process of deacidification in the light (ie. transport of malate out of the vacuole and decarboxylation by malic enzyme). It therefore appeared that the activity of PEP carboxylase *in vivo* was not directly affected by the sodium nutrition.

Instead, Boag (1976) suggested that sodium was perhaps involved in either the efficient photosynthetic assimilation of the endogenous CO_2 produced by decarboxylation in the light, or in the metabolism of the three carbon compound (ie. pyruvate) produced throughout decarboxylation. These suggestions were consistent with the observed reduction in dry-weight of sodium-deficient CAM plants grown under conditions where they are more dependent upon the "C_4-line" option.

Accordingly, sodium might be implicated in the formation or activation of pyruvate, P_i dikinase: an enzyme, present in both C_4 and certain CAM species, which is responsible for the conversion of pyruvate to PEP. In *B. tubiflorum*, a CAM plant having high activities of NADP-ME decarboxylase, this is the first step in the gluconeogenic conservation of the decarboxylation product (pyruvate) as carbohydrate

(Kluge and Osmond 1971, Holtum and Osmond 1981, Osmond and Holtum 1981).

1.3 POSSIBLE METABOLIC ROLES OF SODIUM IN C_4 PLANTS

While there is only limited experimental evidence as to the metabolic consequences of sodium-deficiency in higher plants, there has been considerable speculation as to its specific role in plant metabolism. To date, sodium has been implicated in a variety of reaction processes within the C_4 pathway. Five suggested roles are identified in the schematic presentation of the C_4 pathway shown in Figure 1.01 and are discussed below.

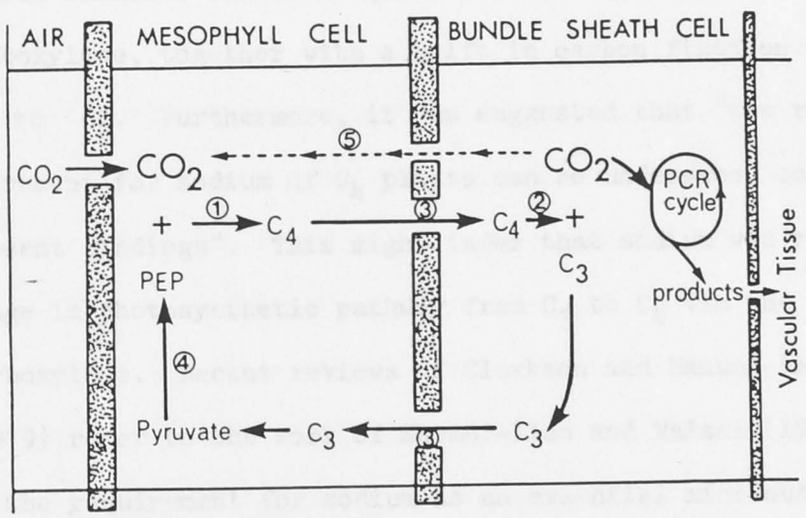


Figure 1.01 A simplified scheme showing the essential biochemical features of C_4 pathway photosynthesis and identifying suggested metabolic roles for sodium in these plants. These are:

1 The formation or activation of PEP carboxylase.

2 Activation of certain decarboxylase enzymes.

3 A $[Na^+ + K^+]$-ATPase system implicated in the active inter-cellular transport of metabolites during C_4 photosynthesis.

4 The activation or formation of enzymes involved in the conversion of pyruvate to phosphoenolpyruvate.

5 Increased back-diffusion of CO_2 in sodium-deficient plants resulting in decreased photosynthetic efficiency ie. that the bundle-sheath compartment of sodium-deficient C_4 plants is "leakier".

1.3.1 The formation or activation of PEP carboxylase

Several workers have suggested that sodium is responsible for the synthesis or activation of PEP carboxylase (Shomer-Ilan and Waisel 1973, Hewitt 1979, Clarkson and Hanson 1980). Shomer-Ilan and Waisel (1973) discussed the effects of sodium chloride on the balance between the C_3 and C_4 pathways of photosynthesis. Increased activities of PEP-carboxylase in young leaves of the C_4 plant *Aeleuropus litoralis*, following application of 100 mM NaCl to the culture medium, together with wholly qualitative descriptions of the distribution of radiocarbon among photosynthetic intermediates prompted these authors to conclude that: sodium chloride caused a "qualitative or quantitative" induction of PEP carboxylase, together with a shift in carbon fixation pathway from C_3 to C_4. Furthermore, it was suggested that "the relatively high requirement for sodium of C_4 plants can be understood on the basis of our present findings". This might infer that sodium was responsible for a change in photosynthetic pathway from C_3 to C_4 via the induction of PEP carboxylase. Recent reviews of Clarkson and Hanson (1980) and Hewitt (1979) refer to the work of Shomer-Ilan and Waisel (1973) and associate the requirement for sodium as an essential micronutrient element with the probable synthesis or activation of PEP carboxylase.

Subsequently, Downton and Törökfalvy (1975) re-examined the influence of sodium chloride on the pathway of photosynthesis in *A.litoralis*. These workers demonstrated that leaves from both high (100 mM NaCl) and low salt status plants did not vary in their photosynthetic pathways. Although application of sodium chloride can alter the photosynthetic pathway of certain species, from a conventional C_3 pathway to a mixture of CAM and C_3 photosynthetic (Winter and von Willert 1972, Ting and Hanscom 1977), this is primarily a response to water stress (Winter and Lüttge 1976, Winter *et al* 1981).

Moreover, preliminary studies with sodium-deficient C_4 plants (Boag and Brownell 1979) refute the suggestions of Shomer-Ilan and Waisel (1973). The high activities of PEP carboxylase in sodium-deficient C_4 plants (Holtum 1975, Brownell 1979) and diurnal patterns of acidification and deacidification in sodium-deficient CAM plants (Boag 1976) would indicate that sodium does not affect the formation or *in vivo* activity of this enzyme. Furthermore, sodium did not stimulate the activity of PEP carboxylase in a low-sodium assay medium containing 0.2 mM sodium (M.D. Hatch, personal communication).

1.3.2 Activation of C_4-acid decarboxylases

Recently, Hewitt (1979) has suggested that sodium is directly involved in the activation of decarboxylase enzymes in C_4 plants. This suggestion was based on the specific requirement for sodium in the activation of certain bacterial OAA-decarboxylases (Stern 1967, O'Brien *et al* 1969) and the observed increases in both anaerobic carbon dioxide production and respiratory oxygen uptake during recovery from sodium-deficiency in *Atriplex* (Brownell and Jackman 1966). In particular, Hewitt indicated that the specific requirement for sodium may be additionally linked with a specific requirement for manganese in the C_4 decarboxylation reaction catalysed by NAD-malic enzyme.

However, the marked respiratory response during recovery from sodium-deficiency is not a general response for C_4 plants (Mill 1977) and a requirement for sodium has been shown for C_4 species having the NADP-ME decarboxylase system which is activated by magnesium or manganese (Brownell and Crossland 1972). Furthermore, from results presented in a following chapter (Chapter 2, Section 2.3.2) and cited by Brownell (1979), it is clear that sodium is generally required by C_4 plants irrespective of the decarboxylase system involved. The

possibility that sodium is specifically required for the activation of all three decarboxylase enzymes, having different intracellular locations, is most unlikely.

1.3.3 A $[Na^+ + K^+]$-ATPase system implicated in the active intercellular transport of metabolites during C_4 photosynthesis

At the present time, concentration gradients of metabolites are considered sufficient to permit the rapid intercellular flux of C_4-photosynthetic intermediates on the basis of diffusion alone (Osmond and Smith 1976, Hatch and Osmond 1976). Recently, Raghavendra and Das (1977, 1978) have reported the occurence of a $[Na^+ + K^+]$ ATPase in the leaves of C_4 plants, with activities five- to ten-fold higher than those measured in the leaves of two C_3 species examined. On the basis of histochemical studies, ^{14}C-labelling experiments with leaves following infiltration with ouabain and measurements of aspartate-dependent alanine production in leaf discs these authors suggested that the $[Na^+ + K^+]$ ATPase was involved in the active transport of C_4 acids from the mesophyll into the bundle sheath. However, these experimental observations do not provide unequivocal evidence for a physiological role of the $[Na^+ + K^+]$ ATPase in the active transport of C_4 acids during steady-state photosynthesis. Furthermore, similarly high activities of $[Na^+ + K^+]$ ATPase were not detected in the leaves of other C_4 species (M.D. Hatch, personal communication).

Recognising that $[Na^+ + K^+]$ ATPases occur in C_3 species (Hansson and Kylin 1969, Karlsson and Kylin 1974), which do not require sodium as an essential micronutrient, and that the concentrations of ions required for maximum activation of $[Na^+ + K^+]$ ATPases are high, in comparison with the concentrations of sodium in the tissues of C_4 plants receiving sufficient sodium for maximum growth (Brownell 1979),

further suggests that the requirement for sodium is not directly linked to the activation of ATPases.

1.3.4 The activation or formation of enzymes involved in conversion of pyruvate to PEP ie. pyruvate, P_i dikinase, adenylate kinase and pyrophosphatase

Whereas considerable diversification exists in the decarboxylation phase of the C_4 pathway, the initial steps of the C_4 cycle (ie. primary fixation of CO_2 and the regeneration of phosphoenolpyruvate) are similar in all C_4 species. Thus, four enzymes have a common and critical role in all C_4 species, are highly active in C_4 but not C_3 plants and are also active in the CAM plant *B. tubiflorum*. These are: PEP carboxylase, pyruvate, P_i dikinase, adenylate kinase and pyrophosphatase (Hatch 1976a,b, Hatch and Osmond 1976, Osmond and Holtum 1981). A direct effect of sodium on either the activation, or formation of one of these enzymes might therefore explain the specific requirement for this element in these plants.

Comprehensive discussions of the activation of enzyme catalysed reactions by monovalent cations have been presented by Evans and Sorger (1966), Suelter (1970) and Evans and Wildes (1971). The following generalisations were made by Evans and Sorger (1966): (i) a wide variety of enzyme reactions of microbial, animal and plant origin are activated by monovalent cations; (ii) usually enzymes activated by K^+ are also activated by Rb^+ or NH_4^+, but are activated little by Na^+ and not at all by Li^+; (iii) usually enzymes activated by Na^+ are also activated by Li^+, but are activated much less or not at all by K^+, Rb^+ or NH_4^+; (iv) the molar concentrations of K^+, Rb^+ or NH_4^+ required for maximum enzyme activity are high ie. activation constant, K_A, approaching 0.01 M in most cases.

Although enzymes specifically activated by sodium have been described in micro-organisms (Stern 1967, O'Brien *et al* 1969), no higher plant enzymes are known to be specifically activated by this element (Evans and Sorger 1966, Suelter 1970). In particular, no activation effect of sodium was observed on pyruvate, P_i dikinase or adenylate kinase in a low-sodium assay medium containing 0.0002 M (4.6 ppm, 0.2 mol m^{-3}) sodium (M.D. Hatch, personal communication). These results, together with the observation that the concentrations of monovalent cations that are required for maximum enzyme activities frequently exceed the concentrations of sodium in tissues of plants receiving enough sodium for normal growth (Brownell 1979), do not suggest the direct involvement of this element in the activation of key C_4 enzymes.

The specific activities of pyruvate, P_i dikinase, adenylate kinase and pyrophosphatase have not been measured in sodium-deficient C_4 plants.

1.3.5 Increased back-diffusion of CO_2 in sodium-deficient plants resulting in decreased photosynthetic efficiency ie. that the bundle-sheath compartment of sodium-deficient plants is leakier

Essentially, the combination of biochemical and anatomical adaptations seen in C_4 species serve as a mechanism for transporting CO_2 to the bundle sheath cells, and for concentrating this CO_2 in the vicinity of RuP_2 carboxylase. Two physiological consequences of this process are seen in the increased water use efficiency, and in the effective elimination of photorespiration in C_4 plants (Hatch 1971, Hatch 1976a,b, Hatch and Osmond 1976, Edwards and Huber 1981).

Following the suggestion by Boag (1976), that sodium-deficiency might affect the efficient refixation of CO_2 produced in the light during CAM, it seems valid to hypothesise that sodium-deficiency could

be affecting the re-fixation of CO_2 in the bundle sheath of C_4 plants. Perhaps a higher proportion of the CO_2 released in decarboxylation "leaks" out of the bundle-sheath compartment and mixes with the CO_2 being fixed by PEP carboxylase. This would result in a substantial decrease in the photosynthetic efficiency of the sodium-deficient C_4 plants and could contribute towards the reduced dry weight yields observed in these plants. Photosynthetic efficiency of sodium-deficient C_4 plants could be further diminished, as a decrease in the $[CO_2/O_2]$ ratio within the bundle sheath during steady-state photosynthesis may result in enhanced fixation of O_2 by RuP_2 oxygenase.

The experiments described in the following chapters investigate various aspects of the photosynthetic carbon metabolism of sodium-deficient and control C_4 plants. In particular, two specific hypotheses concerning the essential role of sodium in C_4 photosynthesis and outlined in Sections 1.3.4 and 1.3.5, are examined.

CHAPTER 2

GROWTH OF PLANTS UNDER LOW-SODIUM CULTURE CONDITIONS

2.1 INTRODUCTION

In order to investigate C_4 photosynthesis in sodium-deficient plants it is necessary to obtain clear-cut signs of sodium deficiency in experimental plants. In *Atriplex vesicaria* the minimum concentration of sodium required for maximum growth is approximately 0.05 mol m^{-3} (1.2 ppm) (Brownell 1965), while the addition of a sodium salt at 0.1 mol m^{-3} (2.3 ppm) to the culture solution is sufficient to support normal growth in other C_4 species (Brownell and Crossland 1972). Due to the ubiquity of sodium, special methods must be employed to achieve and maintain low-sodium culture conditions. These low-sodium culture techniques have been discussed in detail in a recent review by Brownell (1979). The methods used to obtain sodium-deficient plants, throughout this study, were essentially those of Brownell (1965) and are described in this chapter.

The species which were used throughout this study were chosen according to the following criteria. In the first instance, representative species were selected from each of the three C_4 decarboxylation sub-groups: NADP-malic enzyme, NAD-malic enzyme and PCK-type plants (Gutierrez *et al.* 1974, Hatch *et al.* 1975). Of the C_4 species previously shown to require sodium (Brownell and Crossland 1972) none possess the PCK-type decarboxylation system and a requirement for sodium is shown for several PCK-type C_4 species in this chapter. The

second criteria was that sodium-deficient experimental plants should be suitable for leaf gas exchange studies, enzyme extraction, protoplast and organelle isolation and ultrastructural examination. While much of the previous work with sodium-deficient plants has been carried out on *Atriplex* spp (Brownell 1965, Brownell and Jackman 1966), the diminutive yields of sodium-deficient *Atriplex* (e.g. in the case of *A. vesicaria*, the fresh weight yield of sodium-deficient plants was only 0.135 g per plant after forty-eight days (Brownell 1965)) argued against their use as experimental plants in this study. Instead, C_4 species belonging to the Poaceae were selected for the experiments described in following chapters. Such species are readily cultured under low-sodium conditions and exhibit significant growth responses to added sodium (Brownell and Crossland 1972), are suitable for gas exchange experiments (Ku and Edwards 1978a), are amenable to the successful isolation of photosynthetically functional protoplasts and organelles (Huber and Edwards 1975a, b) and, by virtue of their simple leaf anatomy, are suitable for ultrastructural examination (Hatch *et al.* 1975).

This chapter describes the growth responses to sodium, for those C_4 species used throughout this study. This includes determinations of fresh weight/dry weight ratios, shoot/root ratios, total chlorophyll and chlorophyll $^{a}/b$ ratios, specific leaf areas and the root and shoot concentrations of sodium and other mineral elements for both sodium-deficient and control plants. In addition a survey of the carbon isotope discrimination ratios ($\delta^{13}C$) of sodium-deficient and control plants from a range of C_4 species is presented.

2.2 MATERIALS AND METHODS

2.2.1 Sources of seeds

Seeds of *Amaranthus edulis* Speg. and *Kochia childsii* Hort. were obtained commercially. Seeds of *Atriplex inflata* Fv. M., *Atriplex nummularia* Lindl., and *Atriplex vesicaria* Heward were received from Dr. Malcolm Nobs while seeds of *Digitaria sanguinalis* (L) Scop., *Echinochloa crus-galli* var. frumentaceae (Roxb) and *Panicum milioides* Nees ex Trin. were received from Dr. Paul Hattersley. Seeds of *Chloris barbata* Swartz., *Chloris gayana* Kumpth., *Eleusine indica* (L) Gaertn., and *Panicum maximum* Jacq. were collected locally in Townsville, Nth. Queensland.

2.2.2 Growth regimes

Plants were grown under one of three growth regimes. These were

(A) in a controlled environment chamber under the following conditions: 16 hours light (500-600 $\mu Em^{-2}\ s^{-1}$ (400-700 nm))at $27^{o}C$ - 8 hours darkness at $19^{o}C$; or

(B) in a controlled environment chamber under the following conditions: 16 hours light (180-210 $\mu Em^{-2}\ s^{-1}$ (400-700 nm)) at $33^{o}C$ - 8 hours darkness at $21^{o}C$; or

(C) in a naturally illuminated glasshouse under full sunlight, midday irradiance being in excess of 2000 $\mu Em^{-2}\ s^{-1}$ (400-700 nm). Air temperature was controlled at $32^{o}C$ during the day and $18^{o}C$ at night.

2.2.3 Culture apparatus

Plants were grown hydroponically in sodium-free polyethylene culture vessels of 2-litre capacity. Light was excluded from the culture solution

and roots of the plants by wrapping tar paper, black on the inner side and reflective on the outside, around each vessel. Vessels had covers of grey Perspex which held four evenly spaced plants which were secured by cotton wool previously washed in many changes of silica-distilled water. The cotton wool was held in place by split corks made from polyethylene tubing.

Air for culture aeration was passed through a Whatman Gamma-12 in-line filter (retention efficiency 0.3 μm) and cultures were continuously aerated by means of a centrally placed silica-ware or sodium-free plastic tubes which reached to the bottom of the culture vessels.

Seeds were washed in many changes of silica-distilled water and then germinated on nylon gauze sewn onto a circlet of polyethylene tubing which was supported by polystyrene legs in a circular vessel of sodium-free plastic material. A hood of transparent plastic covered this vessel to prevent contamination and at the same time provided a humid environment which facilitated germination of smaller seeds.

Seeds were germinated in half-strength culture solution, and seedlings were selected for uniformity and transplanted when about 1.5 cm in height.

2.2.4 Composition of culture solution

The basal culture solution, composed of salts amenable to purification procedures was that of Brownell (1965). The composition of the culture solution expressed in μmol l^{-1} was as follows:

$Ca(NO_3)_2$, 4,000; $MgSO_4$, 1,000; KNO_3, 5,000; $(NH_4)_2HPO_4$, 1,000; KH_2PO_4, 1,000; H_3BO_3, 46; $MnCl_2.4H_2O$, 9.1; $CuSO_4.5H_2O$, 0.31; $ZnSO_4.7H_2O$, 0.76; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.1; NH_4Cl, 350. Iron

was supplied as the ferric ammonium EDTA at 90 μmol l^{-1} in the basal culture solution.

Sodium chloride was supplied to appropriate cultures to give a final concentration of 0.1 mol m^{-3} (2.3 ppm). These cultures are designated as the control treatment (+Na).

2.2.5 Preparation of low sodium salts

The methods used in the preparation of nutrient salts containing small amounts of sodium are those of Brownell (1965) with some modifications.

All water was silica-distilled and contained less than 0.009μmol l^{-1} sodium. The recrystallisation of salts and the redistillation of nitric acid, sulphuric acid and ammonium hydroxide were carried out in silicaware. Hydrochloric acid was prepared by bubbling hydrogen chloride gas into silica-distilled water. Stock solutions were stored in sodium-free plastic containers.

Solutions of manganese chloride, zinc sulphate, copper sulphate and ammonium molybdate were made up from A.R. grade salts without purification. Ammonium chloride was formed by the addition of ammonium hydroxide to an equivalent amount of hydrochloric acid. Boric acid, free of sodium, was gratefully received from Dr. P.F. Brownell.

Magnesium sulphate, potassium dihydrogen phosphate and potassium nitrate were recrystallised up to six times. Diammonium phosphate was purified by facilitating crystallisation through the addition of absolute ethanol to a saturated aqueous solution. This procedure was repreated up to five times.

Calcium nitrate was obtained by first forming calcium salicylate from calcium carbonate and salicylic acid. The calcium salicylate was then recrystallised six times, dried in an oven and then oxidised in a

muffle furnace (12 hrs, 500°C). Finally the oxide was dissolved in low-sodium nitric acid.

Iron was supplied as ferric ammonium EDTA and was prepared by the method of Jacobsen (1951). However ammonium hydroxide was used instead of potassium hydroxide. Ferrous sulphate was recrystallised six times from solutions acidified by the addition of sulphuric acid and the resulting crystals were then dried at 50°C. The EDTA was first dissolved in 2N ammonium hydroxide and then precipitated by the addition of 2N hydrochloric acid. This procedure was repeated five times and the resultant precipitate washed with silica-distilled water and dried in an oven at 50°C.

Sodium was routinely monitored by atomic absorption spectrophotometry using a Varian Techtron Model AA6.

2.2.6 Fresh weight and dry weight determinations.

At harvests, the tops of plants were removed by cutting the hypocotyl at the level of the top of the split cork and were weighed immediately to obtain their fresh weight. Roots were removed from the culture vessels, dried between cleansing tissues and then dried in paper bags or well washed aluminium foil trays in a well ventilated oven at 80°. After 72 hours plant material was cooled in a desiccator at room temperature and the dry weights obtained.

2.2.7 Leaf area determination

Leaf area was measured with a Lambda leaf area meter (Model LI-3000) immediately after leaves had been excised from the plant.

2.2.8 Chlorophyll determination

Chlorophyll was determined by extracting a measured amount of leaf tissue with 80% acetone in the dark at 0 - 5°C. Total chlorophyll, chlorophyll a and chlorophyll b were calculated using the equations of Mackinney (1941).

2.2.9 Estimation of root and shoot mineral concentrations

Concentrations of Ca, Mg, K, Na, Fe, Mn, Zn and Cu were determined by atomic absorption spectrophotometry using a Varian Techtron Atomic Absorption Spectrophotometer Model AA6.

Plant material, oven-dried to constant weight in aluminium foil trays, was crushed by hand (covered with well-washed plastic surgical glove) and then weighed into small silica-ware crucibles (i.e. approximately 0.3 g per sample). Samples were further disintegrated by maceration with a stainless steel spatula after addition of a small amount of liquid nitrogen - this was found to minimise charring in subsequent ashing.

Samples were then ashed for 16 hours in a muffle furnace. The temperature was slowly raised over an initial 10 hour period to a maximum of 420°C. Samples were digested in 7.5 ml of low-sodium 3N HCl over a steam bath (1½ hours), filtered through pre-washed Whatman No. 54 filter paper, made up to 25 ml and then stored in sodium-free plastic containers. Aliquots were then taken and diluted for determination of individual mineral concentrations. To suppress interference in the determination of calcium and magnesium, aliquots were diluted to contain Sr, K and Na at 1000, 500 and 200 $\mu g\ l^{-1}$ respectively. Sample blanks, minus plant material, contained no detectable sodium.

For the determination of total nitrogen and total phosphorous in

plant material, subsamples (100 mg dry wt) of homogenised root or shoot material were digested in 5 ml of digestion solution which contained 100 ml H_2SO_4 (98% w/w), 1 g selenium powder and 100 g of K_2SO_4. After allowing plant material to cold-digest for one hour, digestion tubes were placed on a temperature-programmable aluminium block digester which was programmed as follows:

$40^{o}C$ (20 min), $120^{o}C$ (20 min), $200^{o}C$ (30 min), $350^{o}C$ (120 min).

The resultant clear solutions were cooled and diluted to 100 ml with distilled water. Sub-samples of the digest were analysed for total N and total P using a Technicon Auto Analyzer II (Technicon Inst., Corp., New York).

2.2.10 $\delta^{13}C$ determinations

Samples of oven-dried shoot material were used for the determination of carbon isotope composition. After combustion, the CO_2 was collected on a freezing train and analysed with a Micromass 602D mass-spectrometer. The ratio of ^{13}C to ^{12}C is expressed as a $\delta^{13}C$ value ($^{o}/oo$), with respect to a standardised plant material which was calibrated against standard limestones (Osmond *et al.* 1979), where

$$\delta^{13}C(^{o}/oo) = \left(\frac{^{13}C/^{12}C \text{ sample}}{^{13}C/^{12}C \text{ standard}} - 1\right) \times 1000$$

2.3 RESULTS AND DISCUSSION

2.3.1 Purification of the basal culture solution

The amounts of sodium contributed to the culture solution as impurities of the purified component salts are given in Table 2.01. The total amount of sodium contributed as impurities of component salts was about 0.077 $\mu mol\ l^{-1}$. This level is comparable to that obtained by Brownell (1965), where sodium contributed by component salts was reduced

Table 2.01 Sodium contributed to the basal culture solution as impurities of component salts

Salt	Concentration of salt in culture solution (μM)	Sodium contributed by component salts (μmol/21 culture)
Potassium nitrate	5,000	0.0252
Calcium nitrate	4,000	0.0443
Potassium dihydrogen phosphate	1,000	0.0260
Diammonium phosphate	1,000	0.0217
Magnesium sulphate	1,000	0.0035
Ammonium chloride	350	0.0007
Ferric ammonium ethylene diamine tetra acetate	90	0.0217
Boric acid	46	0.0007
Manganese chloride [a]	9.1	0.0069
Zinc sulphate	0.76	0.0027
Copper sulphate	0.31	0.0001
Ammonium molybdate	0.10	0.0001
Total sodium in culture due to sodium impurities of all component salts		0.1536 (i.e. 0.0018ppm)

[a] In growth experiments with *E. crus-galli* var. frumentaceae (i.e. *E. utilis*) manganese was supplied at one tenth of the concentration in the basal culture solution. Growth experiments outlined in Appendix A.4 suggested that this species was particularly susceptible to manganese toxity.

from 4.6 µmol l^{-1}, where untreated analytical reagents were used, down to 0.069 µmol l^{-1} after purification.

2.3.2 Growth responses of various species to sodium

Yield responses of various plants to sodium are shown in Table 2.02 and illustrated in Figure 2.01. A requirement for sodium had not been previously shown for the following C_4 species: *D. sanguinalis*, *E. indica*, *C. barbata*, *C. gayana* and *A. edulis*. These results confirm the observation that sodium is generally essential for species having the C_4 pathway (Brownell and Crossland 1972). The growth responses to sodium obtained in these experiments for *E. crus-galli* (synonymous with *E. utilis*) and *K.childsii* are similar to those reported by Brownell and Crossland (1972). The species listed in Table 2.02 include representative species from each of the three C_4 decarboxylation sub-groups, i.e. *D. sanguinalis*, *K. childsii* and *E. crus-galli* are NADP-ME-type C_4 species, *E. indica* and *A. edulis* are NAD-ME-type C_4 species, while *C. barbata*, *C. gayana* and *P. maximum* are PCK-type C_4 species (Gutierrez *et al.* 1974, Hatch *et al.* 1975).

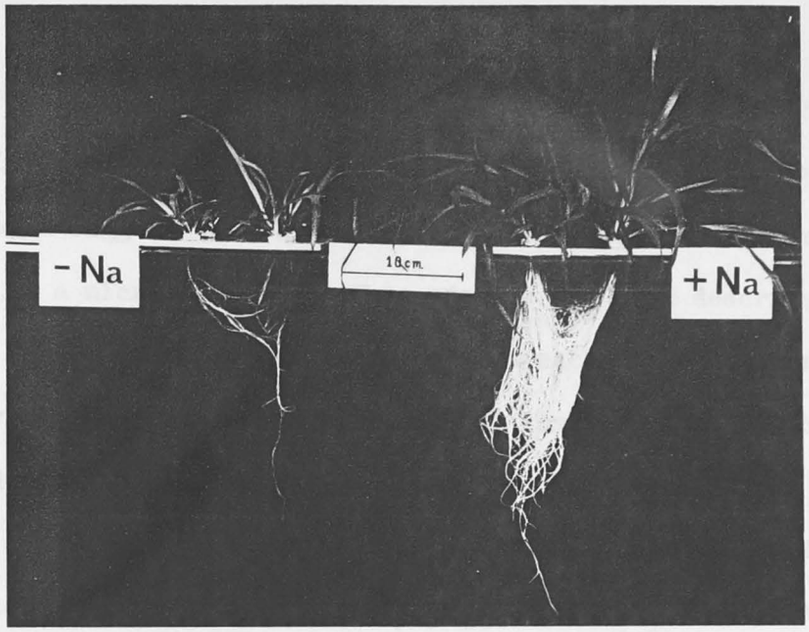
Plants of the above species grown in "sodium free" cultures, i.e. containing less than 0.077 µmol l^{-1} sodium as an impurity, made little growth compared to those supplied with sodium and were characterised by general leaf chlorosis (Figure 2.01, Table 2.03). In *Atriplex* species and in *K. childsii* sodium-deficient plants developed distinct necrotic patches on the tips and margins of leaves similar to those described by Brownell (1965). While *P. maximum* showed only a marginal growth response to sodium (Table 2.02) those plants which were grown in cultures not receiving sodium exhibited leaf chlorosis. While the amount of sodium in the seeds of this species was not determined, it is possible that these might have contributed significant amounts of sodium to the plants, which

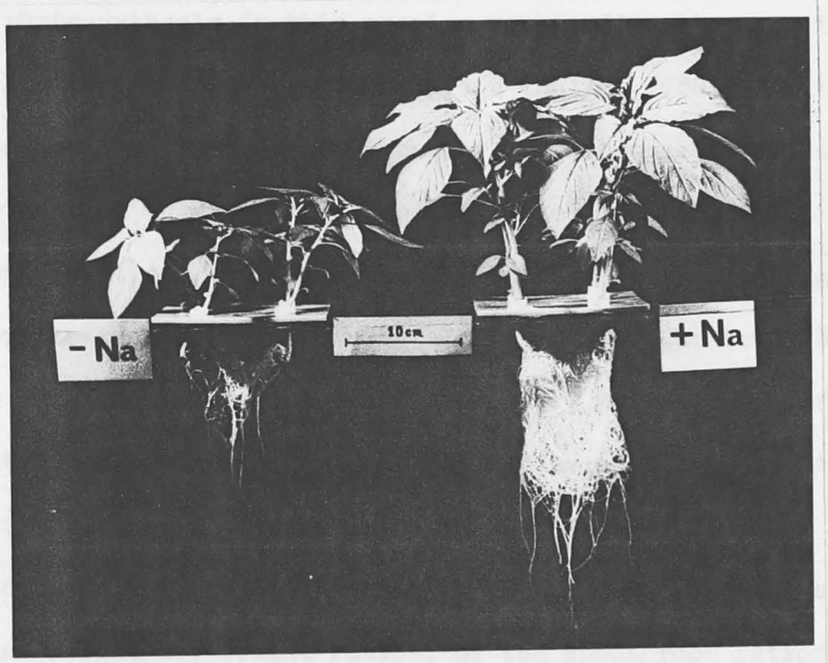
Table 2.02 Yield responses of various plants to sodium. No added sodium (-Na) and 0.1 mol m^{-3} NaCl (+Na)

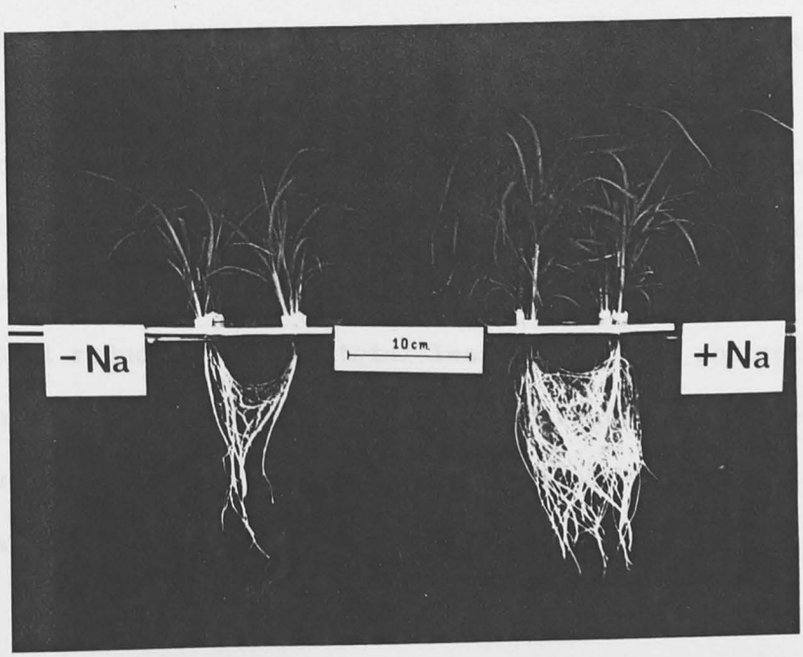
Species and treatment		[e] Age at harvest (days)	Yield (g d.w./plant)	[f] Significance of difference %	Fresh wt / Dry wt	Shoots (d.w.) / Roots (d.w.)
[a] *Echinochloa crus-galli* var. frumentacea (Roxb.) (Japanese millet)	+Na	24	1.165	0.1	13.8	1.6
	-Na		0.455		10.3	2.3
[a] *Digitaria sanguinalis* (L.) Scop. (Crabgrass)	+Na	23	1.422	0.1	10.6	3.2
	-Na		0.638		12.4	3.5
[b] *Eleusine indica* (L.) Gaertn. (Crowsfoot grass)	+Na	21	0.435	0.1	10.1	5.3
	-Na		0.221		11.2	5.2
[c] *Chloris barbata* Swartz (Purple top Chloris)	+Na	23	0.238	0.1	10.6	2.5
	-Na		0.071		10.5	2.5
[c] *Chloris gayana* Kumpth. (Rhodes grass)	+Na	21	0.270	0.1	8.9	5.2
	-Na		0.110		8.9	5.1
[c] *Panicum maximum* Jacq. (Guinea grass)	+Na	17	0.427	N.S.	-	-
	-Na		0.335			
[d] *Panicum milioides* Nees ex trin.	+Na	50	0.533	N.S.	5.1	3.93
	-Na		0.539		5.0	4.10
[b] *Amaranthus edulis* Speg.	+Na	29	2.238	0.1	9.8	1.21
	-Na		0.556		12.2	1.74
[a] *Kochia childsii* Hort.	+Na	21	0.410	0.1	13.4	3.7
	-Na		0.090		12.3	3.8

a NADP-ME type; b NAD-ME type; c PEP-CK type; d "C_3-C_4 Intermediate"; e In subsequent experiments ages of plants were no more than 2 - 3 days older; f Students t-test.

Figure 2.01 Comparison between sodium-deficient (-Na) and control plants (+Na) of *Eleusine indica* (TOP), *Amaranthus edulis* (CENTRE) and *Chloris barbata* (BOTTOM).


- Na
18 cm
+ Na


- Na
10 cm
+ Na


- Na
10 cm
+ Na

would result in a limited growth response to sodium. Plants of *P. milioides* did not respond to the sodium treatment (Table 2.02) and this would suggest that this species does not have a requirement for sodium as a micronutrient. This species has been described as a "C_3-C_4 intermediate" on the basis of gas exchange characteristics, leaf anatomy and the possession of a reduced complement of C_4 enzymes (Quebedeaux and Chollet 1977, Rathnam and Chollet 1980). While the nature of "C_4-like" photosynthesis in *P. milioides* is not fully understood, it would appear that the bulk of the carbon assimilated during photosynthesis in this species passes directly into the PCR cycle (Kestler *et al.* 1975, Rathnam and Chollet 1980). Although *P. milioides* does possess the capacity for increased C_4 dicarboxylic acid metabolism relative to C_3 species, the differentiation of the component reactions and compartmentation of individual enzymes is not analagous to that seen in C_4 plants (Rathnam and Chollet 1980). The absence of a growth response to sodium in *P. milioides* reinforces the correlation between a requirement for sodium as a micronutrient and the possession of the C_4 pathway of photosynthesis.

Sodium deficiency did not result in substantial changes in either the fresh weight/dry weight ratios, or the shoot/root ratios of those C_4 plants examined (Table 2.02). The unaltered shoot/root ratios would imply that sodium deficiency does not result in, and is not the result of, a change in the ratio of photosynthate allocation to photosynthetic versus non-photosynthetic tissues in these species.

Typically, sodium-deficient C_4 plants are characterized by a substantial reduction in the total leaf chlorophyll content, which is accompanied by an increase in the specific leaf area (Table 2.03). The increased specific leaf area was consistent with the visual observation that leaves of sodium-deficient plants were thinner than the control

Table 2.03 Comparisons of specific leaf area, total chlorophyll and chlorophyll [a]/b ratios of leaves from sodium-deficient (-Na) and control plants (+Na) of various C_4 species[d].

		Specific leaf area ($cm^2 g^{-1}$ fresh wt)	Total chlorophyll ($mg.m^{-2}$)	Chlorophyll [a]/b
E. crus-galli [ae]	+Na	53.4(7.2)	390(40)	3.7(0.2)
	-Na	69.0(3.3)	150(20)	3.8(0.3)
D. sanguinalis [ae]	+Na	48.2(1.1)	540(30)	3.6(0.2)
	-Na	61.1(2.8)	310(30)	3.7(0.2)
E. indica [be]	+Na	43.4(1.8)	530(20)	3.7(0.2)
	-Na	57.2(2.8)	240(10)	3.8(0.1)
C. barbata [ce]	+Na	55.3(3.6)	470(50)	3.3(0.2)
	-Na	68.0(2.4)	290(30)	3.2(0.2)
C. gayana [ce]	+Na	57.2(4.5)	470(50)	3.3(0.3)
	-Na	64.6(3.7)	290(30)	3.3(0.2)
A. edulis [be]	+Na	39.7(1.0)	390(30)	4.3(0.3)
	-Na	49.5(2.0)	240(10)	4.1(0.3)
K. childsii [af]	+Na	31.4(2.5)	260(20)	3.8(0.1)
	-Na	36.0(3.1)	60(10)	3.8(0.2)
A. nummularia [bf]	+Na	24.5(2.2)	580(30)	3.1(0.3)
	-Na	31.7(3.4)	180(20)	3.5(0.2)
A. inflata [bf]	+Na	24.0(1.5)	580(30)	3.6(0.2)
	-Na	21.3(2.8)	190(10)	4.0(0.3)

[a] NADP-ME-type

[b] NAD-ME-type

[c] PCK-type

[d] each value is the mean of three independent determinations and standard deviations are given in parentheses

[e] plants were grown in a naturally illuminated glasshouse (Section 2.2.2C)

[f] plants were grown in a growth cabinet (Section 2.2.2A)

leaves (see also Chapter 6, Section 6.3.1). Furthermore, sodium deficiency does not result in any consistent changes in the total leaf chl ^{a}/b ratios (Table 2.03). Tentative interpretation of these chl ^{a}/b ratios would suggest that there is no substantial alteration in either the distribution of chlorophyll between the mesophyll and bundle sheath compartments, or the energy requirements (e.g. the relative contributions of cyclic *vs* non-cyclic electron flow) of these compartments in sodium-deficient leaves. For example, dimorphic chloroplasts of NADP-ME-type C_4 plants have different pigment compositions as the bundle sheath chloroplasts have decreased amounts of chl b relative to chl a, i.e. a higher chl ^{a}/b ratio compared to the mesophyll chloroplasts (Mayne *et al.* 1974, Ku *et al.* 1974, Anderson 1980). In the NADP-ME-types species shown in Table 2.03, the total leaf chl ^{a}/b ratios of sodium-deficient plants are similar to those of the controls, and suggest that the distribution of chlorophyll between the mesophyll and bundle sheath is not altered.

2.3.3 The concentrations of sodium and other mineral nutrients in sodium-deficient plants

The concentrations of sodium, and other mineral nutrients, were measured in shoot and root fractions from sodium-deficient and control plants of *E. crus-galli*, *E. indica* and *C. barbata* (Table 2.04). Concentrations of sodium in the sodium-deficient and control shoots of these species ranged from 0.65 - 1.29 μmol g^{-1} dry wt (15 - 30 ppm) and from 4.25 - 44.61 μmol g^{-1} dry wt (100 - 1025 ppm), respectively. Such concentrations are considerably lower than those obtained in *Atriplex* species grown with similar concentrations of sodium in their culture solutions (Brownell 1965, Brownell and Jackman 1966) and

Table 2.04 Mineral concentrations of shoot (a) and root (b) material from sodium-deficient (-Na) and control plants (+Na) of *E. crus-galli*, *E. indica* and *C. barbata*.

Species and treatment			Mineral element ($\mu mol\ g^{-1}$ Dry wt)					($mmol\ g^{-1}$ Dry wt)		
			Na	Mn	Cu	Zn	Fe	K	Ca	Mg
E. crus-galli	+Na	Shoots[a]	4.25(0.79)	1.39(0.16)	0.16(0.03)	0.78(0.04)	0.78(0.12)	2.17(0.11)	0.15(0.01)	0.18(0.004)
		Roots[b]	17.10	1.59	0.19	0.29	17.2	1.85	0.15	0.28
	-Na	Shoots	0.65(0.13)	2.38(0.09)	0.37(0.10)	0.96(0.11)	0.39(0.12)	1.89(0.10)	0.11(0.01)	0.16(0.01)
		Roots	0.88	13.20	0.99	1.30	11.75	1.81	0.40	0.35
E. indica	+Na	Shoots	4.99(0.18)	2.08(0.23)	0.14(0.02)	0.19(0.02)	0.88(0.18)	1.14(0.05)	0.38(0.04)	0.15(0.01)
		Roots	14.43	2.06	0.11	0.25	57.54	1.54	0.09	0.13
	-Na	Shoots	1.05(0.08)	3.51(0.17)	0.21(0.02)	0.51(0.04)	0.49(0.03)	1.68(0.04)	0.45(0.05)	0.17(0.02)
		Roots	3.57	7.56	1.19	1.41	12.20	1.28	0.38	0.11
C. barbata	+Na	Shoots	44.61(3.55)	1.95(0.10)	0.14(0.02)	0.28(0.05)	3.19(0.52)	1.39(0.04)	0.30(0.03)	0.16(0.01)
		Roots	14.65	1.61	0.11	0.19	17.80	1.25	0.12	0.07
	-Na	Shoots	1.29(0.22)	1.92(0.10)	0.36(0.09)	0.66(0.06)	0.54(0.04)	1.88(0.08)	0.21(0.01)	0.13(0.01)
		Roots	2.14	22.87	0.54	1.24	6.97	1.72	0.26	0.12

[a] Shoots - values given are the means of three independent samples for all species and treatments. Standard deviation given in brackets.

[b] Roots - value given is that of a single sample for all species and treatments.

demonstrate that a requirement for sodium is not correllated with an ability to accumulate sodium at high concentrations in the tissues from low substrate levels (see also Table 2.05). An estimate of the total sodium uptake by plants from the culture solutions for *E. crus-galli*, *E. indica* and *C. barbata*, using the sodium concentrations from Table 2.04 and estimates of dry weight yield/culture and shoot/root ratios for the same population of plants (N.B. for *E. indica* and *C. barbata* these are different plant populations from those shown in Table 2.02) is given in Table 2.05. This analysis provides an assessment of the degree of sodium contamination occurring throughout the course of one growth cycle in the naturally illuminated glasshouse (Section 2.2.2c) in which the majority of experimental plants in this study were grown. From Table 2.05 it is evident that 0.83 - 1.26 µmol sodium was recovered from sodium-deficient cultures, which contained approximately 0.172 µmol sodium as an impurity, suggesting that over a period of about 20 days these cultures received as much as 1 µmol sodium through contamination. This is likely to be an overestimate as a proportion of this sodium would have been contributed by the seeds. For example, Brownell (1968) found that the amounts of sodium per seed after washing was variable and ranged from 0.001 µmol per seed for tomato up to 0.27 µmol per seed for *A. vesicaria*. While the degree of sodium contamination in these experiments could have been reduced by growing plants in a specially constructed glasshouse, designed to prevent contamination of plants by sodium from the atmosphere (Brownell and Wood 1957, Brownell 1965), I concluded that clear-cut signs of sodium-deficiency could be consistently obtained in this conventional glass-house. However, I would stress that Canberra's inland location was ideal as compared to the relatively large accessions of sodium occurring in coastal areas which do not permit the culture of sodium-deficient plants in conventional glass-houses (Brownell 1965).

Table 2.05 Estimations of total sodium uptake from the culture solutions by sodium-deficient (-Na) and control plants (+Na) of *E. crus-galli*, *E. indica* and *C. barbata*

Species and treatment		Total dry wt per culture (g)	Shoot:Root Ratio(D.W.)	Na in Shoots (µmol)	Na in Roots (µmol)	Total sodium[c] in plant material (µmol)
E. crus-galli	+Na[a]	4.66	1.6	11.7	29.3	41.0
	-Na[b]	1.82	2.3	0.79	0.47	1.26
E. indica	+Na	5.24	3.3	19.2	16.8	36.0
	-Na	0.84	4.0	0.67	0.58	1.25
C. barbata	+Na	3.95	2.4	119.27	16.29	135.56
	-Na	0.57	2.8	0.52	0.31	0.83

[a] Control culture solutions contained approximately 200 µmol sodium/2 l culture

[b] Sodium-deficient culture solution contained approximately 0.172 µmol sodium/2 l culture

[c] There was no detectable sodium remaining in the "sodium free" culture solution upon harvest

Table 2.06 Total nitrogen and phosphorous content of sodium-deficient (-Na) and control plants (+Na) of *A. edulis* and *K.childsii*[a]

Species and treatment			Total N (% dry wt)	Total P (% dry wt)	N:P ratio
A. edulis	+Na	Shoots	5.25(0.13)	1.03(0.05)	5.12
		Roots	4.93(0.11)	0.93(0.02)	5.30
	-Na	Shoots	6.00(0.10)	0.89(0.07)	6.74
		Roots	4.99(0.04)	1.13(0.01)	4.42
K. childsii	+Na	Shoots	4.07(0.14)	0.74(0.02)	5.50
		Roots	3.04(0.07)	0.91(0.03)	3.34
	-Na	Shoots	5.16(0.04)	0.74(0.04)	6.97
		Roots	2.89(0.05)	0.86(0.02)	3.36

[a] Values given are the means of three independent samples and standards deviations are given in brackets

The concentrations of other mineral elements in sodium-deficient and control plants were different in some instances (Table 2.04). For example, the concentrations of iron were higher in the shoots of control plants when compared to sodium-deficient plants. Conversely the roots of sodium-deficient plants were characterised by increased manganese concentrations. However, there were no consistent differences between sodium-deficient and control treatments in the levels of potassium, calcium or magnesium. While the concentrations of various mineral elements are different in sodium-deficient plants, the magnitudes of such differences do not suggest that sodium-deficiency is related to the deleterious accumulation, or depletion of another mineral element. However, more extensive experiments would be necessary to determine whether there exists any direct relationship between sodium nutrition and the uptake of other mineral nutrients.

The total nitrogen and phosphorous contents of sodium-deficient and control plants of *A. edulis* and *K. childsii* are shown in Table 2.06. There were no substantial differences in the total nitrogen and phosphorous contents of either roots, or shoots of sodium-deficient and control plants of these species. Similarly, Brownell and Jackman (1966) determined that the total nitrogen contents of sodium-deficient and control leaves of *Atriplex* species were similar. While sodium-deficient leaves of *Atriplex* species had relatively higher ratios of 80% ethanol-soluble nitrogen to total nitrogen, this ratio did not change rapidly during recovery from sodium deficiency and Brownell and Jackman (1966) concluded that the long-term effect of sodium in decreasing the ratio of soluble nitrogen to total nitrogen was indirect.

2.3.4 $\delta^{13}C$ values of sodium-deficient and control plants

During photosynthesis plants discriminate against the heavier

Table 2.07 Carbon isotope ratios ($\delta^{13}C$) for sodium-deficient (-Na) and control plants (+Na) of several C_4 species

Species	$\delta^{13}C(^{o}/oo)$ Treatment	
	+Na	-Na
E. crus-galli[a]	-11.5, -10.7	-8.0, -8.0
E. indica[a]	-12.2, -12.1	-10.5, -10.6
C. barbata[ab]	-11.8,-12.4[b]/-12.2,-12.2[a]	-13.1,-13.7[b]/-11.0,-10.8[a]
A. edulis[a]	-10.4, -10.6	-8.2, -8.1
K. childsii[b]	-13.3, -13.3	-13.3, -13.3
A. spongiosa[b]	-11.2, -11.7	-10.2
A. inflata[b]	-12.9	-13.9
A. nummularia[b]	-12.5	-13.5

[a] Plants grown in naturally illuminated glass-house (Section 2.2.2C)
[b] Plants grown in a growth cabinet (Section 2.2.2A)

carbon isotope ^{13}C and C_3, C_4 and CAM plants can be distinguished on the basis of their carbon isotope composition. The mean $\delta^{13}C$ value for C_4 species is more positive than that of C_3 species (Bender 1971, Smith and Epstein 1971, Troughton *et al.* 1974), while CAM plants express intermediate values becoming more positive as the plant shifts from predominately light ("C_3-like") to predominately dark ("C_4-like") CO_2 fixation (Osmond *et al.* 1973, Lerman 1975). The $\delta^{13}C$ values of sodium-deficient and control plants shown in Table 2.07 are consistent with photosynthetic CO_2 assimilation *via* the C_4 pathway.

While differences between the $\delta^{13}C$ values of sodium-deficient and control plants are evident, a consistent trend is only seen in those plants grown in a naturally illuminated glasshouse. Under these conditions sodium-deficient plants show more positive $\delta^{13}C$ values, with differences between means ranging from $1.3^{o}/oo$ - $3.1^{o}/oo$. When plants

were grown in a controlled environment chamber there were no consistent differences between the $\delta^{13}C$ values of sodium-deficient and control plants (Table 2.07). These observations could simply reflect inter-specific variation in the effect of sodium deficiency on the plant carbon isotope composition. However, $\delta^{13}C$ values obtained for *C. barbata*, where determinations were made on plant material from both growth regimes, could suggest a possible relationship between growth environment and the effect of sodium deficiency on plant carbon isotope composition. The following discussion addresses the possible implications of those substantial and consistent changes in $\delta^{13}C$ values obtained in sodium-deficient plants grown under the glasshouse regime.

The principal components of isotope discrimination during photosynthetic CO_2 assimilation relate to the diffusion of CO_2, the interconversion of CO_2 and HCO_3^-, the enzymatic incorporation of CO_2 or HCO_3^- by RuP_2 carboxylase and PEP carboxylase respectively, and respiratory processes. Recently, quantitative analyses of these fractionation processes during photosynthetic CO_2 assimilation have been presented for both C_3 (O'Leary 1981, Farquhar *et al.* 1981) and C_4 plants (Farquhar 1981, O'Leary 1981, Hattersley 1981). The more positive $\delta^{13}C$ values of sodium-deficient plants suggests, in qualitative terms, that the magnitude of discrimination against ^{13}C by RuP_2 carboxylase during C_4 photosynthesis is lower in these plants. This would imply reduced leakage of CO_2 during decarboxylation and refixation in the bundle sheath compartment of sodium-deficient C_4 plants (Farquhar 1981, O'Leary 1981, Hattersley 1981). In a following section (Chapter 3 Section 3.3.5, Table 3.04) quantitative estimates of this back-diffusion of CO_2, i.e. leakage from the bundle sheath compartment, are made for sodium-deficient and control plants according to the theoretical equations of Farquhar (1981). These estimates suggest that

something less than a two-fold reduction in back-diffusion of CO_2 would result in the observed differences in $\delta^{13}C$ values. Experimental verification of the probable contribution of this back-diffusive flux in determining the carbon isotope composition of C_4 plants was recently presented by Hattersley (1981). This author measured the $\delta^{13}C$ values of representative species from each of the three C_4 acid decarboxylation sub-groups within the Poaceae. Differences between the means for NADP-ME-types, PCK-types and NAD-ME-types were statistically significant, with the NAD-ME-types expressing the most negative $\delta^{13}C$ values. Hattersley (1981) suggested that increased apoplastic leakage of CO_2 from bundle sheath tissue of NAD-ME-types, which lack the suberized lamella observed in PCK- and NADP-ME-types, may cause these species to exhibit the most negative $\delta^{13}C$ values.

Observed changes in $\delta^{13}C$ values as a result of sodium deficiency might also be related to the relative rates of carboxylative and diffusive processes during photosynthetic CO_2 assimilation in C_4 plants. For example, O'Leary (1981) predicted $\delta^{13}C$ values for C_4 plants in accordance with the following assumptions. In the first instance, if the rate of CO_2 fixation is entirely limited by carboxylation, then predicted $\delta^{13}C$ values are near $0^{o}/oo$. In the second case, if diffusion is limiting while carboxylation is relatively fast, then $\delta^{13}C$ values of about $-11^{o}/oo$ were predicted. Consequently the more positive $\delta^{13}C$ values observed for sodium-deficient plants imply, in terms of O'Leary's model, that diffusive processes are less limiting while carboxylative processes are more limiting during photosynthesis in these plants. Indeed, reduced rates of photosynthetic CO_2 assimilation accompanied by higher intercellular partial pressures of CO_2 in sodium-deficient leaves (Section 3.3.5, Figure 3.06 and 3.07) would suggest that diffusive processes are less limiting in these plants.

Further speculation on the physiological significance of observed changes in the $\delta^{13}C$ values of sodium-deficient plants is not warranted. The $\delta^{13}C$ values of sodium-deficient plants are a clear indication of photosynthetic assimilation of CO_2 *via* the C_4 pathway, while differences in the $\delta^{13}C$ values reflect changes in the stoichiometric relationships of the component processes of discrimination in these plants.

2.4 SUMMARY

Using previously established techniques for the growth of plants under low-sodium culture conditions, a requirement for sodium was shown for representative species from each of the C_4 decarboxylation sub-groups. While sodium-deficient C_4 plants showed greatly reduced dry-weight production and total leaf chlorophyll concentrations, the shoot/root ratios, fresh weight/dry weight ratios and total leaf chlorophyll $^a/_b$ ratios were unaffected by the sodium nutrition.

In those species examined, the concentrations of sodium in the shoots of sodium-deficient and control plants ranged from 0.65-1.29 μmol g^{-1} dry Wt (0.08-0.14 mM on a tissue water basis) and 4.25-44.61 μmol g^{-1} dry Wt (0.41-4.80 mM on a tissue water basis), respectively. The concentrations of other mineral elements in sodium-deficient and control plants suggested that sodium-deficiency does not involve the deleterious accumulation or depletion of another mineral element.

The $\delta^{13}C$ values of sodium-deficient plants were consistent with photosynthetic CO_2 assimilation *via* the C_4 pathway. In several species the $\delta^{13}C$ values of sodium-deficient plants were more positive (less negative) than controls.

CHAPTER 3

GAS EXCHANGE CHARACTERISTICS OF SODIUM-DEFICIENT C_4 PLANTS

3.1 INTRODUCTION

Extensive research has confirmed that physiological properties of net CO_2 exchange in intact leaves of C_3 and C_4 plants relate to the biochemical properties of photosynthetic carbon metabolism. For example, differences in CO_2 response curves (Björkman *et al.* 1971, Osmond *et al.* 1969, Ku and Edwards 1978a, Slatyer 1970) and CO_2 compensation points (Moss 1971, Forrester *et al.* 1966a, 1966b), light response curves (Gifford 1971) and quantum yields (Björkman and Ehleringer 1974, Ehleringer and Björkman 1977), and in the sensitivity of photosynthesis to changing oxygen concentrations (Björkman 1971a, Osmond *et al.* 1969, Ku and Edwards 1977) are seen to reflect, and in part define, fundamental differences in the component processes of photosynthesis in C_3 and C_4 species.

In C_4 plants, photosynthetic CO_2 assimilation is greater at low intercellular $p(CO_2)$ and tends to saturate at lower intercellular $p(CO_2)$ when compared to C_3 plants (Osmond *et al.* 1969, Björkman *et al.* 1971). The CO_2 compensation point of C_4 species is typically about 0 ppm CO_2 and is insensitive to changing O_2 concentration, while that of C_3 species is typically about 47 ppm CO_2 and is linearly dependent upon O_2 concentration (Moss 1971, Forrester *et al.* 1966a, 1966b, Poskuta 1969). While the light-limited rates of photosynthesis

(i.e. quantum yield) of C_3 and C_4 species are approximately the same at 25 to 30°C in normal air (i.e. 330 µbar CO_2, 21%O_2), that of C_3 species is strongly dependent upon O_2 concentration, intercellular p(CO_2) and temperature (Björkman and Ehleringer 1974, Ehleringer and Björkman 1977). In C_3 species the light-saturated rate of photosynthesis, under normal CO_2 concentrations, is markedly inhibited by 21%O_2 while that of C_4 species is unaffected by changing O_2 concentrations (Osmond *et al.* 1969, Björkman 1971a,b). Such differences are consistent with the suggestion that the C_4 pathway is basically a mechanism for transporting CO_2 to the bundle sheath compartment and serves to concentrate CO_2 in the vicinity of RuP_2-carboxylase (Hatch and Osmond 1976), therefore overcoming the inhibition by atmospheric O_2, which is an intrinsic property of the RuP_2-carboxylase-oxygenase enzyme in C_3 and C_4 plants (Lorimer and Andrews 1973). In effect, recent models of photosynthesis summarise such relationships, between the biochemical and photochemical attributes of photosynthesis and leaf gas exchange, in C_3 plants (Hall and Björkman 1975, Farquhar *et al.* 1980) and to a lesser extent in C_4 plants (Berry and Farquhar 1978, Peisker 1979).

Leaf gas exchange has been used extensively to investigate photosynthetic response to mineral deficiency (Nátr 1972, 1975). With the possible exception of potassium, where an induced deficiency resulted in a direct decline in stomatal conductance (Peaslee and Moss 1968), observed changes in net rate of CO_2 assimilation relate to a decline in mesophyll capacity, i.e. non-stomatal components of photosynthesis, in addition to changes in stomatal conductance. As a consequence, recent studies evaluating relationships between mineral nutrition and photosynthesis involve the use of gas exchange in

conjunction with detailed biochemical, photochemical and anatomical measurements (Terry 1976, 1977, 1979, 1980, Terry and Ulrich 1973, Longstreth and Nobel 1980, Medina 1969, 1970. Peoples and Koch 1979, von Caemmerer and Farquhar 1981, Stocking 1975).

To date there have been no experiments investigating leaf gas exchange in relation to sodium deficiency. However, Brownell (1979) presented the hypothesis, based on studies with sodium-deficient CAM plants, that sodium deficiency *per se* might be a consequence of increased "leakiness" between bundle sheath and mesophyll compartments (Section 1.3.5). This notion was supported by the observation that sodium deficiency is less pronounced when plants are grown under high partial pressure of CO_2 (ie. $p(CO_2)$ of 1500 µbar), Brownell (personal communication).

In response to this hypothesis and to further characterise C_4 photosynthesis in sodium-deficient plants this chapter examines the CO_2 compensation point, the dependence of net CO_2 assimilation rate on irradiance and intercellular $p(CO_2)$, the quantum yield, the response of photosynthesis to changing oxygen concentrations and the components of net O_2 exchange in leaves of sodium-deficient and control plants of several C_4 species.

A preliminary report of the work described in this chapter has been published under the following title: C_4 photosynthesis in sodium-deficient plants (T.S. Boag and P.F. Brownell, *Aust. J. Plant Physiol.*, 1979, 6, 431-4).

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Sodium-deficient and control plants of *Amaranthus edulis* Speg.,

Atriplex nummularia Lind., *Kochia childsii* Hort., *Chloris barbata* Swartz, *Digitaria sanguinalis* (L) Scop., *Echinochloa crus-galli* var. frumentaceae (Roxb.) and *Eleusine indica* (L) Gaertn. were obtained using the procedures for germination and growth of plants under low sodium conditions detailed in the previous chapter (see Section 2.2.1 - 7). All plants were grown in a naturally illuminated glass-house under full sunlight (see Section 2.2.2(c)) unless otherwise stated. At the time of the experiments the age of the plants corresponded to those given previously (see Table 2.2.2). In all experiments sodium-deficient and control plants were of the same age and only young fully expanded leaves were used.

3.2.2 Determination of CO_2 compensation point

Compensation point determinations were made using the closed gas exchange system described by Boag and Brownell (1979). A detailed description of this gas exchange system is given in Appendix A.1. With the exception of *K. childsii* all determinations were made on attached leaves. Leaf temperature was maintained at 28°C and all measurements were made at a light intensity of 2 mE m^{-2} s^{-1} (400-700 nm).

3.2.3 Measurement of the rates of CO_2 assimilation and transpiration

Two open gas exchange systems were used to measure the rates of transpiration of water vapour and assimilation of CO_2. These gas exchange systems, while essentially similar, are designated Open System (I) or Open System (II) and are described in Appendices A.2.1 and A.2.2 respectively. In such gas exchange systems the outputs of CO_2 analysers and humidity sensors are continuously recorded. This allows

the calculation of rates of CO_2 and water vapour exchange, stomatal conductance to gaseous diffusion and intercellular partial pressure of CO_2 according to the analysis of gas exchange described in Appendix A.2.3.

Experiments were carried out on attached leaves, during normal photoperiods, and culture solutions were continuously aerated. Leaf temperature and water vapour pressure difference between leaf and air were maintained at 28°C and 20 mbar, respectively. Ambient partial pressure of CO_2, P_a, was taken to be 330 μbar.

Experimental treatments were standardised in the following way. Initially, the plant was exposed to humidity, light intensity, temperature, CO_2 and O_2 concentrations comparable to those under which it was grown (e.g. 20 mbar V.P.D., 2 mE m^{-2} s^{-1}, 28°C, 330 μbar CO_2 and 21% O_2). Only after steady-state rates had been obtained did the experiment proceed. In determining the effect of light intensity on photosynthesis and transpiration, data points were taken as light intensity was sequentially reduced. To determine the effect of CO_2 concentration on photosynthesis, at saturating light intensity, data points were taken as the partial pressure of CO_2 was decreased sequentially. The CO_2 concentration was then returned to ambient and steady-state rates were compared with those initially obtained. Data points were then taken as the CO_2 concentration was increased.

To measure photosynthesis and transpiration under different ambient oxygen concentrations leaves were initially pre-treated as described above. They were then exposed to 2% O_2, returned to 21% O_2, then exposed to 50% O_2 and finally returned to 21% O_2. Ambient partial pressure of CO_2, P_a, was kept at 330 μbar during these measurements and care was taken to provide that steady-state conditions prevailed in each

instance before data points were taken.

To determine the effect of CO_2 concentration on photosynthesis, under different ambient oxygen concentrations (i.e. 2% O_2, 21% O_2 or 50% O_2), leaves were pre-treated as described previously. After steady-state conditions were obtained, the oxygen concentration was adjusted and CO_2 response experiments were carried out according to the standardised procedure.

At the conclusion of each experiment leaf material was excised and estimates of total leaf area and chlorophyll were made.

3.2.4 Estimation of quantum yield

Rates of CO_2 assimilation and transpiration were made under conditions where photosynthesis was light limited. Gas exchange measurements were made using open system (I) (see Appendix A.2.1). At each light intensity several measurements were made.

In order to relate incident light intensity to actual absorbed light, estimates of leaf reflectance and transmittance were made using an Ulbricht integrating sphere. Spectra of leaf-absorptance (400-700 nm) were made using a CAREY-14 Recording Spectrophotometer as the light source for the sphere. Light absorptance values for leaves used in gas exchange experiments were obtained using the xenon lamp, as light source for the sphere, and measurements were made using a quantum sensor (Lambda Instruments).

3.2.5 Measurement of oxygen exchange in leaves in the light

The responses of O_2 exchange (i.e. O_2 production and O_2 uptake) to ambient CO_2 concentration were measured using isotopic techniques with

the closed gas exchange system of Canvin *et al.* (1980). Measurements were made on intact leaves maintained at 28°C and at a light intensity of 2 mE m^{-2} s^{-1}. A detailed description of this gas exchange system is given in Appendix A.1.

3.3 RESULTS AND DISCUSSION

3.3.1 CO_2 compensation point

From Table 3.01 it can be seen that the CO_2 compensation points of those species studied was unaffected by the sodium nutrition. The compensation points of sodium-deficient plants were effectively zero and are consistent with those reported for C_4 plants (Moss 1971, Black 1973).

Table 3.01 The CO_2 compensation points of sodium-deficient (-Na) and control plants (+Na) of several C_4 species.

Species	[a] CO_2 compensation point (µbar CO_2)	
	-Na	+Na
K. childsii [b]	0	1
A. edulis [c]	0	0
C. barbata [c]	0	0
E. indica [c]	0	0

[a] Results are the means of two independent determinations.

[b] Measurements made on excised leaf tissue in 21% O_2 at 35°C; irradiance was 1 mE m^{-2} s^{-1}.

[c] Measurements made on intact leaves in 21% O_2 at 28°C; irradiance was 2 mE m^{-2} s^{-1}.

In addition there does not appear to be any substantial difference between sodium-deficient and control plants of *K. childsii*, in the response of CO_2 compensation point to increasing temperature.

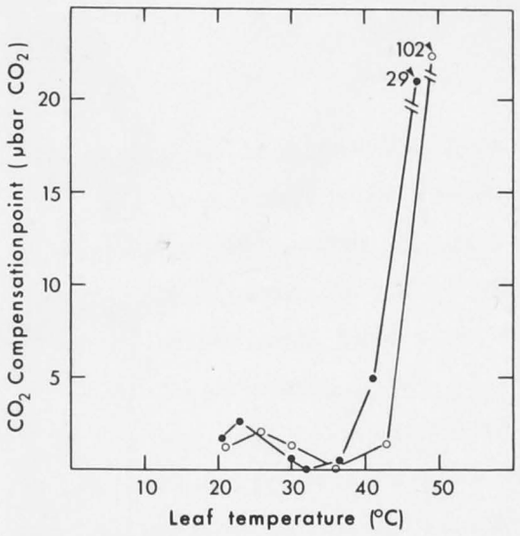


Figure 3.01 Relationship between CO_2 compensation point and leaf temperature in detached leaves of sodium-deficient (o) and control plants (●) of *K. childsii*.

The pronounced increase in compensation point at temperatures in excess of 43°C was accompanied by severe wilting in both deficient and control plants.

3.3.2 The response of CO_2 assimilation to irradiance

The responses of photosynthetic CO_2 assimilation to irradiance for sodium-deficient and control plants of *E. crus-galli*, *E. indica* and *C. barbata*, and *A. edulis* and *A. nummularia* are shown in Figures 3.02 and 3.03 respectively. In each instance the response of CO_2 assimilation to irradiance is shown on a leaf area basis and then replotted on a total leaf chlorophyll basis.

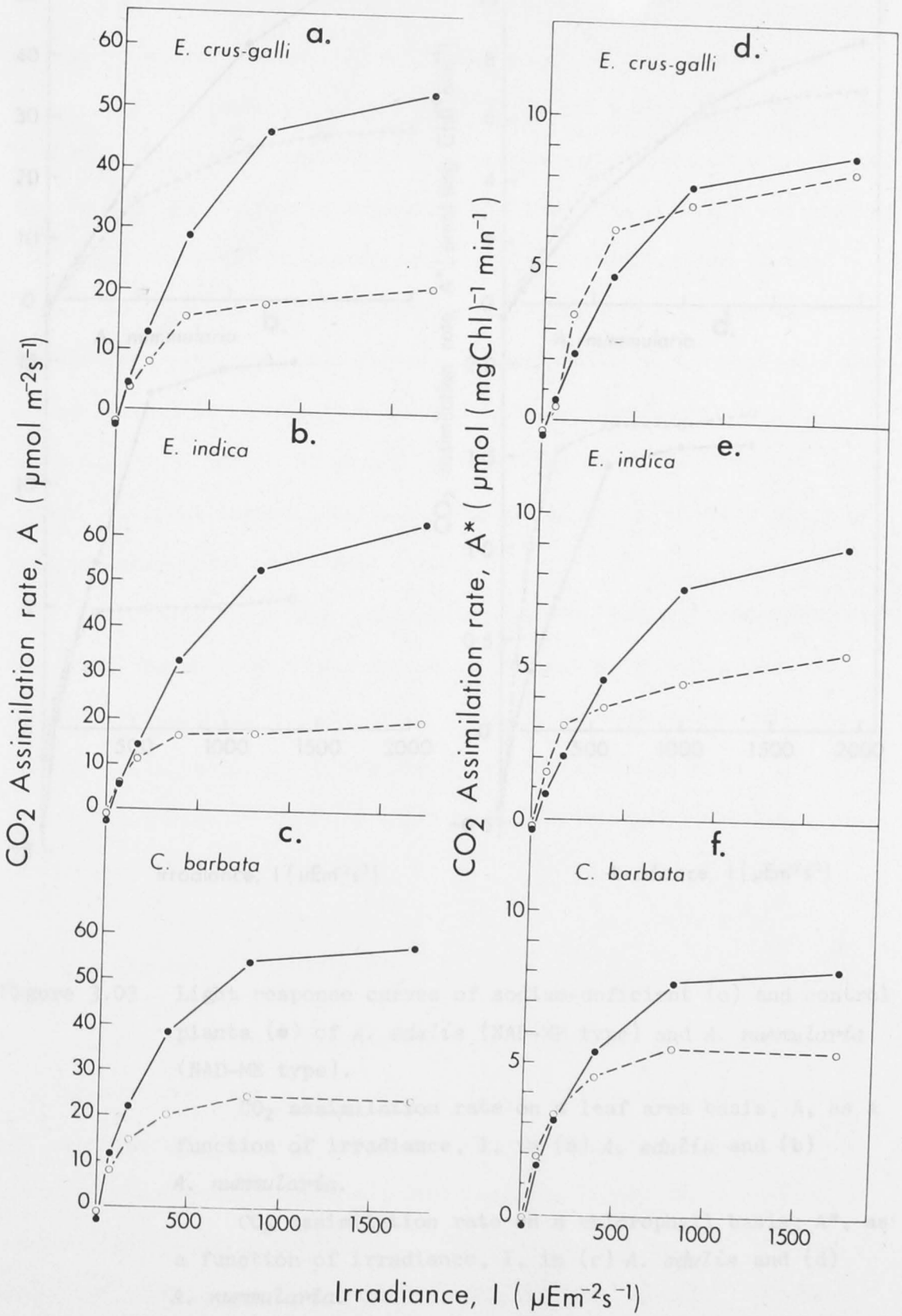
In all species the response of CO_2 assimilation at low irradiance in sodium-deficient plants is comparable to that of controls. However, the light-saturated rate of photosynthesis is substantially reduced in sodium-deficient plants. Furthermore the response of CO_2

Figure 3.02 Light response curves of sodium-deficient (o) and control plants (•) of representative species of the three sub-groups of C_4 plants: *E. crus-galli* (NADP-ME type), *E. indica* (NAD-ME type) and *C. barbata* (PCK-type).

CO_2 assimilation rate on a leaf area basis, A, as a function of irradiance, I, in (a) *E. crus-galli*, (b) *E. indica* and (c) *C. barbata*.

CO_2 assimilation rate on a chlorophyll basis, A*, as a function of irradiance, I, in (d) *E. crus-galli* (e) *E. indica* and (f) *C. barbata*.

Leaf temperature was 28°C, vapour pressure difference was 20 mbar and ambient partial pressure of CO_2 was 330 µbar.


a.
E. crus-galli
b.
E. indica
c.
C. barbata
d.
E. crus-galli
e.
E. indica
f.
C. barbata
CO_2 Assimilation rate, A (μmol $m^{-2}s^{-1}$)
CO_2 Assimilation rate, A* (μmol (mgChl)$^{-1}$ min^{-1})
Irradiance, I ($\mu Em^{-2}s^{-1}$)
0
10
20
30
40
50
60
5
500
1000
1500

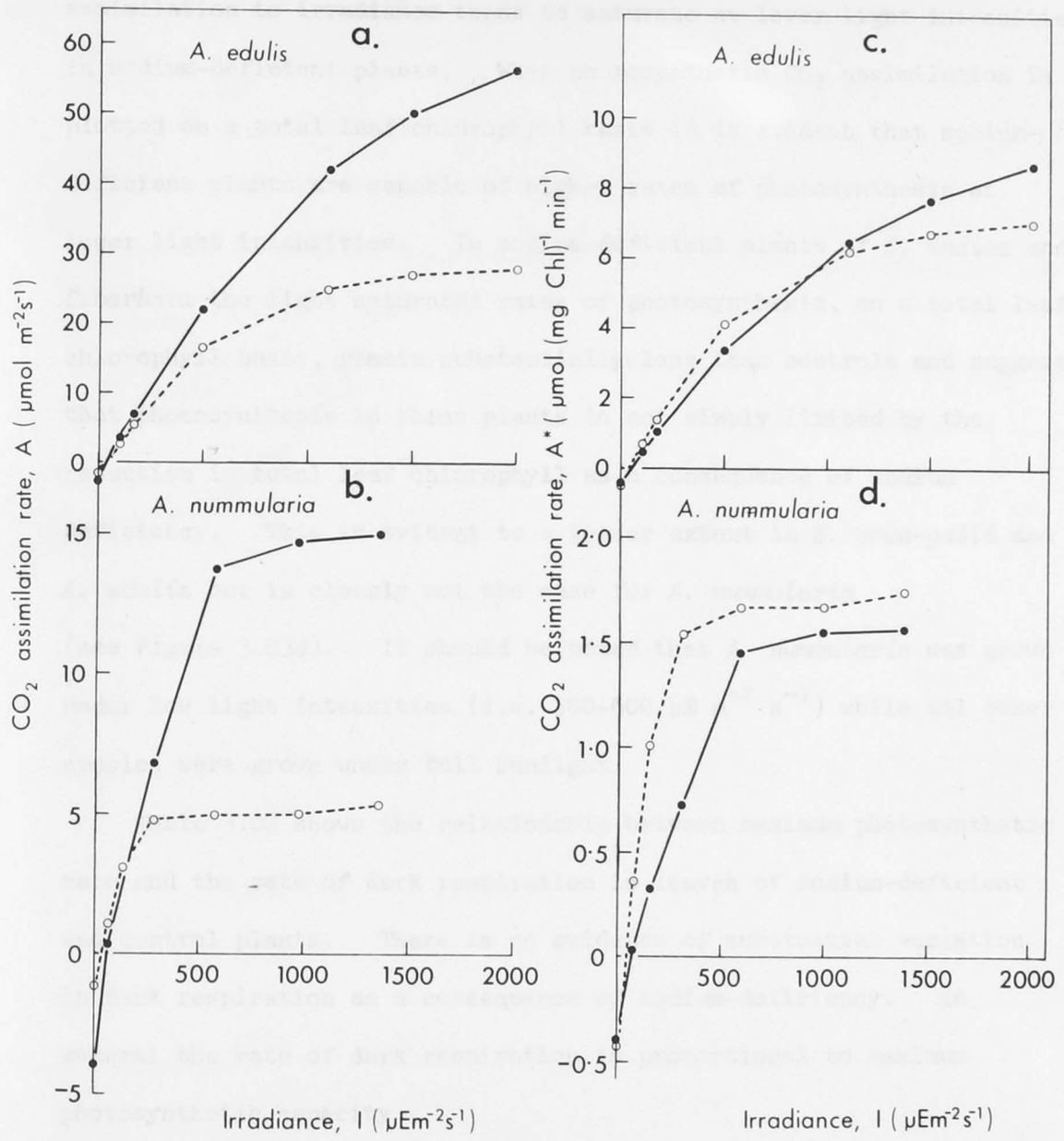


Figure 3.03 Light response curves of sodium-deficient (o) and control plants (●) of *A. edulis* (NAD-ME type) and *A. nummularia* (NAD-ME type).

CO_2 assimilation rate on a leaf area basis, A, as a function of irradiance, I, in (a) *A. edulis* and (b) *A. nummularia*.

CO_2 assimilation rate on a chlorophyll basis, A*, as a function of irradiance, I, in (c) *A. edulis* and (d) *A. nummularia*.

Leaf temperature was 28°C, vapour pressure difference was 20 mbar and ambient partial pressure of CO_2 was 330 μbar.

assimilation to irradiance tends to saturate at lower light intensities in sodium-deficient plants. When photosynthetic CO_2 assimilation is plotted on a total leaf chlorophyll basis it is evident that sodium-deficient plants are capable of higher rates of photosynthesis at lower light intensities. In sodium-deficient plants of *E. indica* and *C.barbata* the light saturated rates of photosynthesis, on a total leaf chlorophyll basis, remain substantially less than controls and suggest that photosynthesis in these plants is not simply limited by the reduction in total leaf chlorophyll as a consequence of sodium deficiency. This is evident to a lesser extent in *E. crus-galli* and *A. edulis* but is clearly not the case for *A. nummularia* (see Figure 3.03d). It should be noted that *A. nummularia* was grown under low light intensities (i.e. 500-600 $\mu E\ m^{-2}\ s^{-1}$) while all other species were grown under full sunlight.

Table 3.02 shows the relationship between maximum photosynthetic rate and the rate of dark respiration in leaves of sodium-deficient and control plants. There is no evidence of substantial variation in dark respiration as a consequence of sodium-deficiency. In general the rate of dark respiration is proportional to maximum photosynthetic capacity.

3.3.3 Quantum yield estimates

Figure 3.04 shows quantum yield estimates for CO_2 uptake in sodium-deficient and control plants of *A. edulis* and *D. sanguinalis*. In both species there are no appreciable differences between sodium-deficient and control plants in the response of net CO_2 assimilation under light-limiting conditions. This is consistent with the earlier observation that apparent quantum yield, as seen in light response

Table 3.02 The relationship between maximum rate of CO_2 assimilation and dark respiration in leaves of sodium-deficient and control plants.

Species and treatment		$A_{max}(A,I)$ [a] ($\mu mol\ m^{-2}\ s^{-1}$)	Dark respiration ($\mu mol\ m^{-2}\ s^{-1}$)	$\frac{A_{max}(A,I)}{\text{Dark respiration}}$
E. crus-galli	+Na	56.10	-1.61	34.84
	-Na	20.61	-1.3	15.85
E. indica	+Na	56.13	-1.80	31.18
	-Na	20.30	-0.72	28.19
C. barbata	+Na	57.1	-2.50	22.84
	-Na	22.2	-1.04	21.35
A. edulis	+Na	56.50	-2.53	22.33
	-Na	28.07	-1.78	15.77
A. nummularia	+Na	15.02	-3.88	3.87
	-Na	5.13	-1.20	4.28

[a] Maximum rates of CO_2 assimilation, $A_{max}(A,I)$, were obtained from light response curves (see Figures 3.02 and 3.03) where P_a = 330 µbar CO_2 and leaf temperature was 28°C.

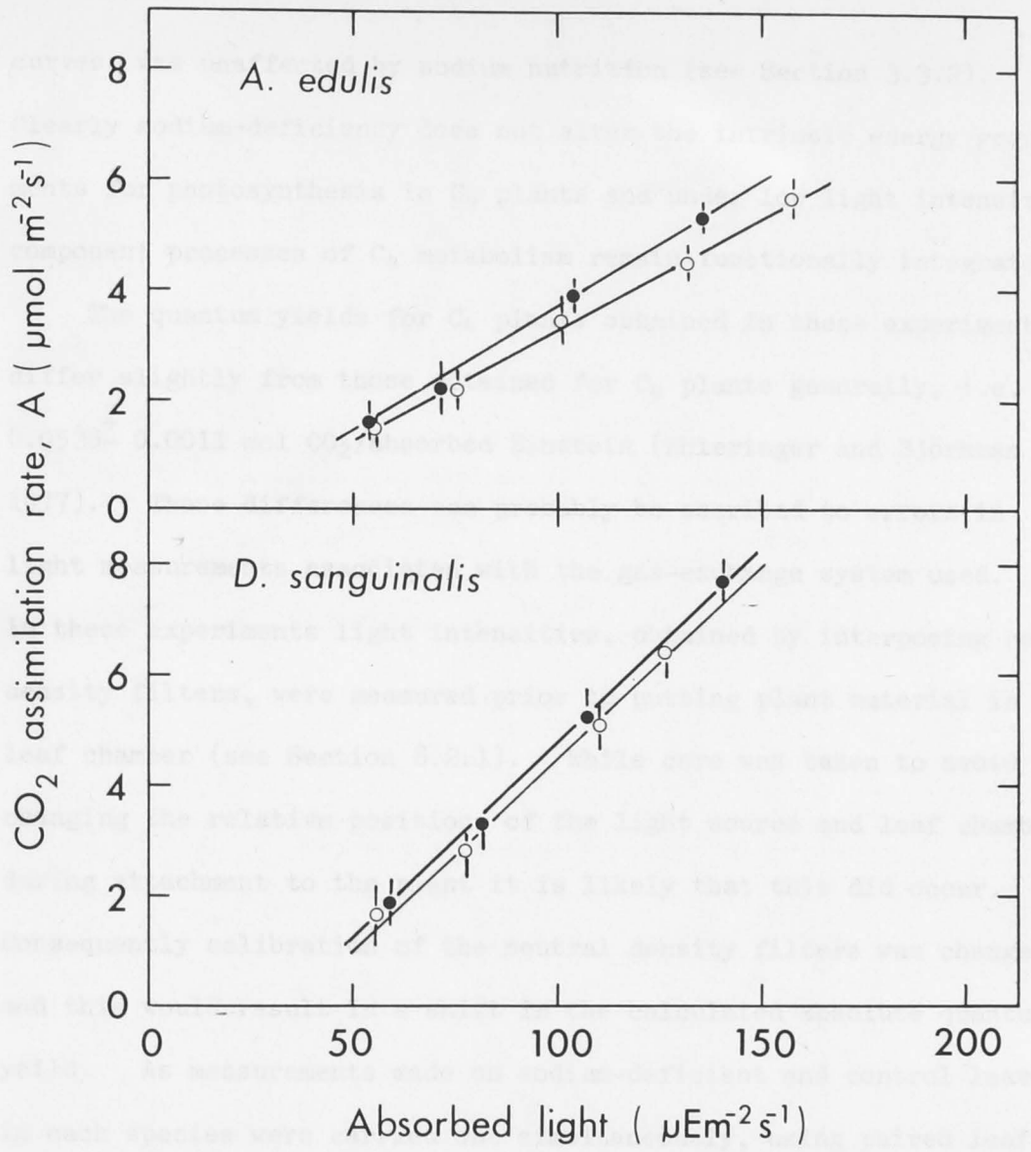


Figure 3.04 Rate of CO_2 assimilation, A, versus absorbed quantum flux in sodium-deficient (o) and control leaves (•) of *A. edulis* (NAD-ME type) and *D. sanguinalis* (NADP-ME type). Each data point is the mean of five steady-state determinations and error bars represent standard deviations.

Linear regressions are:-

A. edulis (+Na); $A = 0.047\ (I) - 0.63$, $r^2 = 0.90$

A. edulis (-Na); $A = 0.042\ (I) - 1.02$, $r^2 = 0.99$

D. sanguinalis (+Na); $A = 0.067\ (I) - 2.32$, $r^2 = 0.99$

D. sanguinalis (-Na); $A = 0.073\ (I) - 2.54$, $r^2 = 0.99$

Leaf temperature was 28°C, vapour pressure difference was 20 mbar and ambient partial pressure of CO_2 was 330 μbar.

curves, was unaffected by sodium nutrition (see Section 3.3.2). Clearly sodium-deficiency does not alter the intrinsic energy requirements for photosynthesis in C_4 plants and under low light intensities component processes of C_4 metabolism remain functionally integrated.

The quantum yields for C_4 plants obtained in these experiments differ slightly from those obtained for C_4 plants generally, i.e. 0.0538 ± 0.0011 mol CO_2/absorbed Einstein (Ehleringer and Björkman 1977). These differences can probably be ascribed to errors in light measurements associated with the gas-exchange system used. In these experiments light intensities, obtained by interposing neutral density filters, were measured prior to putting plant material in the leaf chamber (see Section 8.2.1). While care was taken to avoid changing the relative positions of the light source and leaf chambers during attachment to the plant it is likely that this did occur. Consequently calibration of the neutral density filters was changed and this would result in a shift in the calculated absolute quantum yeild. As measurements made on sodium-deficient and control leaves in each species were carried out simultaneously, using paired leaf chambers, the light calibration within a single experiment is internally consistent.

Leaf absorptance values were lower in leaves of sodium-deficient plants. For example, in *A. edulis* quantum absorptances were 91% and 84%, and in *D. sanguinalis* were 92% and 85% for control and sodium-deficient leaves respectively. Differences in leaf absorptance of control and sodium-deficient leaves are further illustrated by the leaf absorptance spectra shown in Figure 3.05.

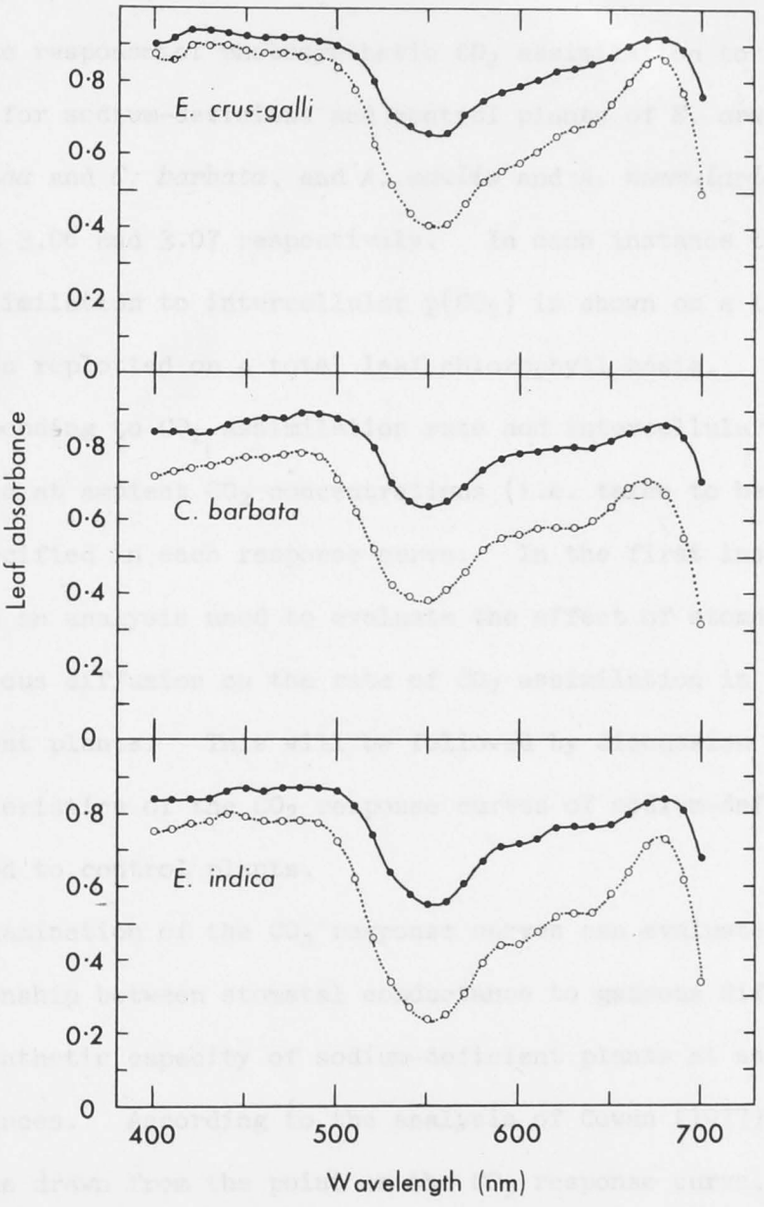


Figure 3.05 Leaf absorptance spectra of sodium-deficient (o) and control plants (●) of *E. crus-galli*, *E. indica* and *C. barbata*.

3.3.4 The response of CO_2 assimilation to intercellular $p(CO_2)$

The responses of photosynthetic CO_2 assimilation to intercellular $p(CO_2)$ for sodium-deficient and control plants of *E. crus-galli*, *E. indica* and *C. barbata*, and *A. edulis* and *A. nummularia* are shown in Figures 3.06 and 3.07 respectively. In each instance the response of CO_2 assimilation to intercellular $p(CO_2)$ is shown on a leaf area basis and then replotted on a total leaf chlorophyll basis. Those values corresponding to CO_2 assimilation rate and intercellular $p(CO_2)$ obtained at ambient CO_2 concentrations (i.e. taken to be 330 μbar CO_2) are specified in each response curve. In the first instance I will discuss an analysis used to evaluate the effect of stomatal conductance to gaseous diffusion on the rate of CO_2 assimilation in sodium-deficient plants. This will be followed by discussion of the kinetic characteristics of the CO_2 response curves of sodium-deficient as compared to control plants.

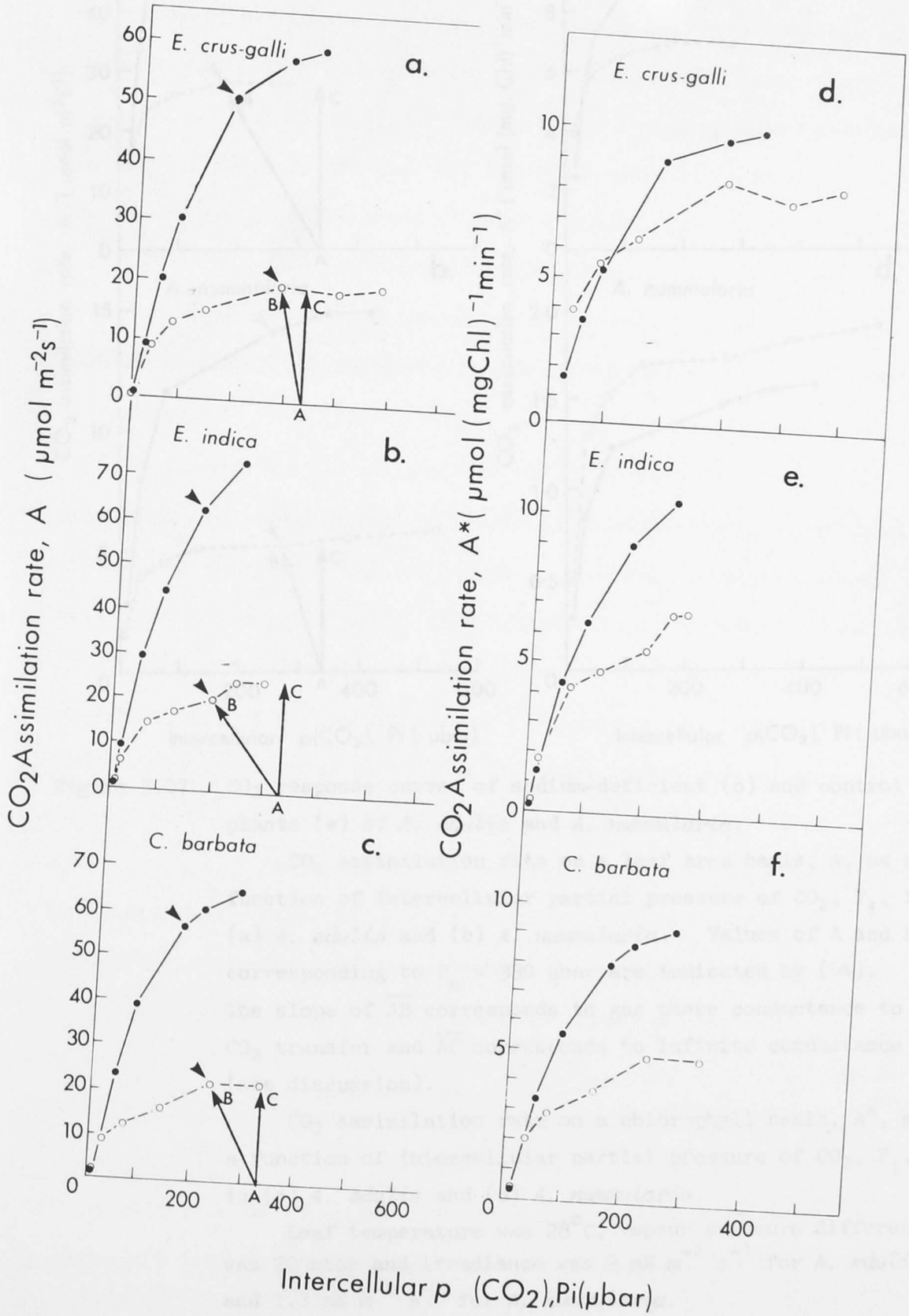
Examination of the CO_2 response curves can evaluate the relationship between stomatal conductance to gaseous diffusion and the photosynthetic capacity of sodium-deficient plants at saturating irradiances. According to the analysis of Cowan (1977) the slope of the line drawn from the point on the CO_2 response curve, obtained at ambient $p(CO_2)$ (i.e. 330 μbar), which intersects the abscissa at ambient $p(CO_2)$, corresponds to the gas phase conductance to CO_2 transfer. In Figures 3.06 and 3.07 this is designated by the interval $\overline{AB}$ with a slope designated $-g^*$ (i.e. g^* is the gas phase conductance to CO_2 transfer, given $-g^* = A/P_a - P_i$). Analogously, the line perpendicular to ambient $p(CO_2)$ corresponds to infinite gas phase conductance to CO_2 transfer and assumes no stomatal or boundary layer limitations,(i.e. intercellular $p(CO_2)$ is equal to ambient $p(CO_2)$).

Figure 3.06 CO_2 response curves of sodium-deficient (o) and control plants (•) of representative species of the three sub-groups of C_4 plants: *E. crus-galli* (NADP-ME type), *E. indica* (NAD-ME type) and *C. barbata* (PCK type).

CO_2 assimilation rate on a leaf area basis, A, as a function of intercellular partial pressure of CO_2, P_i, in (a) *E. crus-galli*, (b)*E. indica* and (c) *C. barbata*. Values of A and P_i corresponding to P_a = 330 µbar are indicated by (▲). The slope of $\overline{AB}$ corresponds to gas phase conductance to CO_2 transfer (i.e. $-g^* = A/P_a - P_i$) and $\overline{AC}$ corresponds to infinite conductance (see discussion).

CO_2 assimilation rate on a chlorophyll basis, A^*, as a function of intercellular partial pressure of CO_2, P_i, in (d) *E. crus-galli*, (e) *E. indica* and (f) *C. barbata*.

Leaf temperature was 28°C, vapour pressure difference was 20 mbar and irradiance was 2 mE m^{-2} s^{-1}.


E. crus-galli
a.
E. indica
b.
C. barbata
c.
E. crus-galli
d.
E. indica
e.
C. barbata
f.
A
B
C
CO_2 Assimilation rate, A (μmol $m^{-2}s^{-1}$)
CO_2 Assimilation rate, A*(μmol (mgChl)$^{-1}$min^{-1})
Intercellular p (CO_2),Pi(μbar)

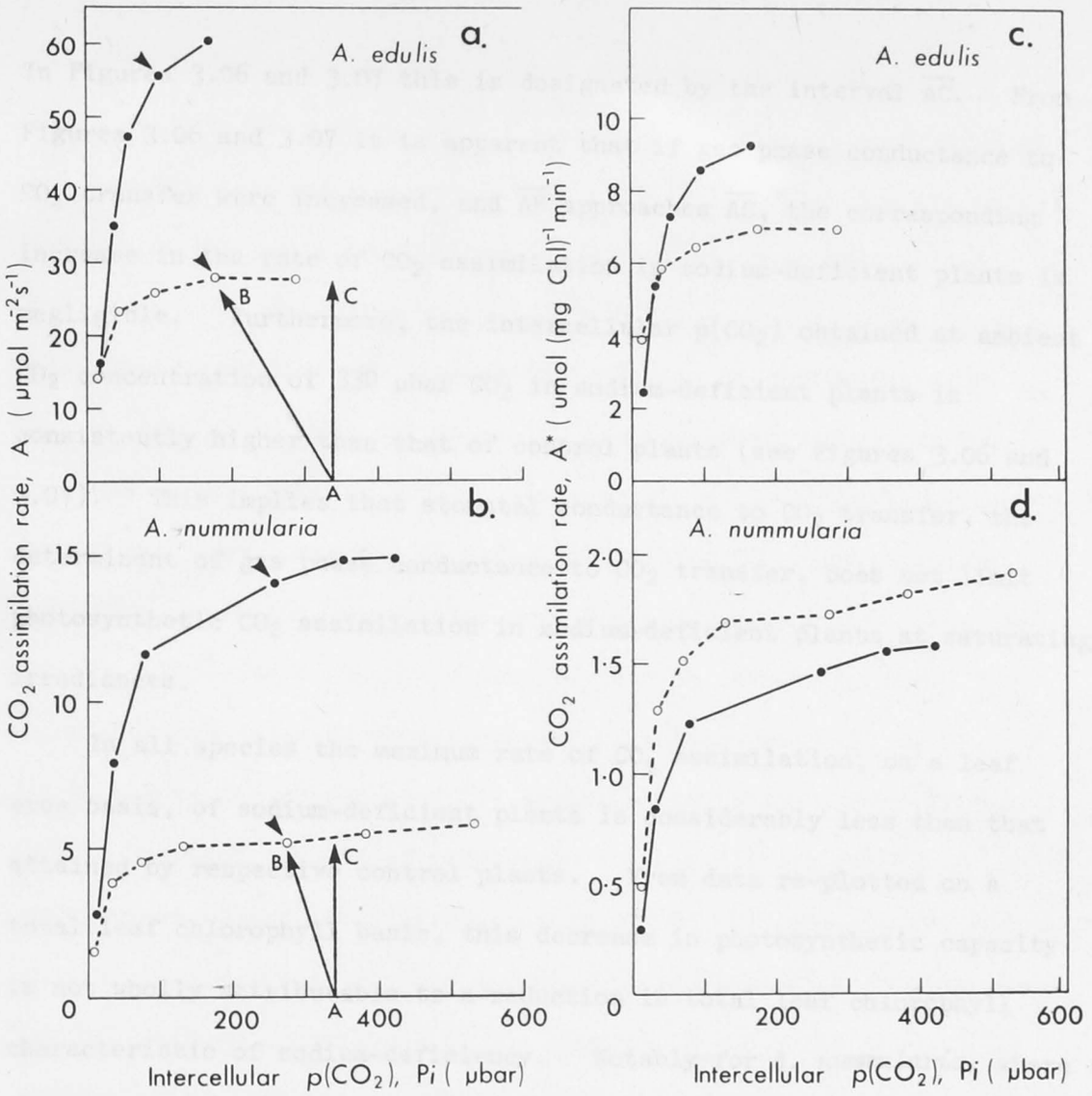


Figure 3.07 CO_2 response curves of sodium-deficient (o) and control plants (•) of *A. edulis* and *A. nummularia*.

CO_2 assimilation rate on a leaf area basis, A, as a function of intercellular partial pressure of CO_2, P_i, in (a) *A. edulis* and (b) *A. nummularia*. Values of A and P_i corresponding to P_a = 330 µbar are indicated by (▲). The slope of $\overline{AB}$ corresponds to gas phase conductance to CO_2 transfer and $\overline{AC}$ corresponds to infinite conductance (see discussion).

CO_2 assimilation rate on a chlorophyll basis, A*, as a function of intercellular partial pressure of CO_2, P_i, in (c) *A. edulis* and (d) *A. nummularia*.

Leaf temperature was 28°C, vapour pressure difference was 20 mbar and irradiance was 2 mE m^{-2} s^{-1} for *A. edulis* and 1.3 mE m^{-2} s^{-1} for *A. nummularia*.

In Figures 3.06 and 3.07 this is designated by the interval $\overline{AC}$. From Figures 3.06 and 3.07 it is apparent that if gas phase conductance to CO_2 transfer were increased, and $\overline{AB}$ approaches $\overline{AC}$, the corresponding increase in the rate of CO_2 assimilation in sodium-deficient plants is negligible. Furthermore, the intercellular $p(CO_2)$ obtained at ambient CO_2 concentration of 330 µbar CO_2 in sodium-deficient plants is consistently higher than that of control plants (see Figures 3.06 and 3.07). This implies that stomatal conductance to CO_2 transfer, the determinant of gas phase conductance to CO_2 transfer, does not limit photosynthetic CO_2 assimilation in sodium-deficient plants at saturating irradiances.

In all species the maximum rate of CO_2 assimilation, on a leaf area basis, of sodium-deficient plants is considerably less than that attained by respective control plants. From data re-plotted on a total leaf chlorophyll basis, this decrease in photosynthetic capacity is not wholly attributable to a reduction in total leaf chlorophyll characteristic of sodium-deficiency. Notably for *A. nummularia*, where plants were grown under relatively low light intensities (i.e. 500-600 $\mu E\ m^{-2}\ s^{-1}$, (400-700 nm)), this is not the case, and on a total leaf chlorophyll basis the sodium-deficient plant is consistently more efficient than corresponding control plant. These observations recapitulate the observed responses of CO_2 assimilation to irradiance in sodium-deficient and control plants of these species (see Section 3.3.3). In all species the initial slopes of the CO_2 response curves for sodium-deficient and control plants are similar and extrapolate to a zero CO_2 compensation point. This observation is consistent with the measured CO_2 compensation points described previously (see Section 3.3.1). However the response of CO_2

assimilation to intercellular $p(CO_2)$ in sodium-deficient plants, while similar to controls at low intercellular $p(CO_2)$, consistently saturate at much lower intercellular $p(CO_2)$, i.e. the apparent half-saturation CO_2 concentration for CO_2 assimilation is substantially less than that of control plants. Such observations suggest that reactions which limit photosynthetic capacity in sodium-deficient plants, while not apparent under low intercellular $p(CO_2)$, or low irradiance (see Section 3.3.3), are exacerbated as photosynthetic CO_2 assimilation is increased by either increasing CO_2 concentrations, or increasing light intensities (see Section 3.3.3).

To what extent can these observations be related to the component processes of photosynthesis of sodium-deficient C_4 plants? In C_4 plants the response of CO_2 assimilation to low intercellular $p(CO_2)$ has been largely attributed to the high levels of PEP carboxylase, the primary carboxylating enzyme in these plants, and the low *in vitro* $K_m(HCO_3^-)$ of this enzyme. This suggestion obtains some support through comparisons of the apparent $K_m(CO_2)$ for the carboxylation reaction *in vivo*, as estimated from the measured response of CO_2 assimilation to intercellular $p(CO_2)$ in intact leaves, with the *in vitro* estimates of $K_m(HCO_3^-)$ for PEP-carboxylase. For example, Ku and Edwards (1978a) estimated the $K_m(CO_2)$ for whole leaf photosynthesis in C_4 plants at 1.6-2.2 μM CO_2 and noted that PEP-carboxylase is reported to have a $K_m(HCO_3^-)$ as low as 20 μM (i.e. at 30°C, pH 7.8 would be in equilibrium with about 0.7 μM CO_2). In addition Peisker (1979) reviewed estimates of the $K_m(CO_2)$ for the carboxylation reaction *in vivo*, derived from the determination of half-saturating intercellular $p(CO_2)$ for CO_2 uptake in intact leaves, and concluded that these were comparable to the lowest published values of *in vitro*

Michaelis constant of PEP carboxylase. However in C_4 plants the response of CO_2 assimilation to higher intercellular $p(CO_2)$, and this would include the region where CO_2 assimilation tends to saturate with increasing CO_2 concentration , is more difficult to interpret.

Given the compartmentation and the complexity of component reactions of the C_4 pathway it is unlikely that CO_2 assimilation by intact leaves should be dictated solely by the kinetic properties of PEP carboxylase. In C_3 plants the component processes determining the response of CO_2 assimilation in intercellular $p(CO_2)$ are better understood. Most recently von Caemmerer and Farquhar (1981) have shown that the response of CO_2 assimilation to low intercellular $p(CO_2)$ is correlated with the *in vitro* activity of RuP_2 carboxylase, while the response to high intercellular $p(CO_2)$ correlated with *in vitro* electron transport capacity (i.e. ascribed by these authors as the primary determinant of RuP_2 regeneration) in the leaves of C_3 plants.

By analogy the response of C_4 photosynthesis to increasing intercellular $p(CO_2)$, in excess of low intercellular $p(CO_2)$ where PEP carboxylase is likely to be the primary determinant, might be related to processes linked to either the flux of metabolites between bundle-sheath and mesophyll compartments (eg. feedback inhibition of the decarboxylase reactions or of PEP carboxylase), or processes involved in the regenerative phases within the bundle-sheath or mesophyll compartments (eg. RuP_2 regeneration and PEP regeneration respectively). Given the reduced levels of RuP_2 carboxylase in C_4 plants, as compared to C_3 plants (Medina 1970, Björkman *et al.*1976), and the relatively high activities of PEP carboxylase, then the

maximum rate of photosynthetic CO_2 assimilation in these plants would presumably be determined by the levels of RuP_2 carboxylase in the leaves of these plants. Unfortunately the significance of such constraints in relation to steady-state C_4 photosynthesis are as yet unresolved.

Anticipating results presented in a following chapter (Chapter 5, Section 5.3.3) will facilitate interpretation of the observed responses to intercellular $p(CO_2)$ in sodium-deficient plants. The activities of PEP-carboxylase and RuP_2 carboxylase were measured *in vitro* for sodium-deficient and control leaves, comparable to those used in gas exchange experiments, of *E. crus-galli*, *E.indica* and *C. barbata*. The activities of PEP-carboxylase and RuP_2 carboxylase (i.e. on a leaf area basis) and the maximum assimilation rate (i.e. as obtained from CO_2 response curves), together with the ratios of PEP-carboxylase: RuP_2 carboxylase, and of experimental maximum assimilation rate: predicted maximum assimilation rate (i.e. if determined by measured *in vitro* RuP_2 capacity) in sodium-deficient and control plants of these species are shown in Table 3.03.

In each of these species the capacity of PEP-carboxylase in sodium-deficient plants is comparable to that of control plants. Consequently the response of CO_2 assimilation to low intercellular $p(CO_2)$ in sodium-deficient plants is similar to that of control plants. However, the estimated RuP_2 carboxylase capacity of sodium-deficient plants is in every instance much less than that of controls. If the maximum photosynthetic capacity of these plants were proportional to the RuP_2 carboxylase capacity of the leaves then these differences are consistent with the depressed rate of photosynthesis in sodium-deficient plants.

However, while the ratios of experimental $A_{max}(A,Pi)$:predicted A_{max} (ie. RuP_2 carboxylase capacity) are near to,

Table 3.03 The experimental maximum assimilation rate obtained from CO_2 response curves (A_{max} (A,Pi)), the activities of PEP-carboxylase (PEP-C) and RuP_2 carboxylase (RuP_2-C) PEP-carboxylase activity:RuP_2 carboxylase activity and experimental maximum assimilation rate (A_{max} (A,Pi)): predicted maximum assimilation rate (i.e. RuP_2-C capacity) in leaves of sodium-deficient and control plants of *E. crus-galli*, *E. indica* and *C. barbata*.

Species and treatment		Experimental [a] A_{max}(A,Pi)	RuP_2-C [b] capacity	PEP-C [b] capacity	PEP-C / RuP_2-C	Exp A_{max}(A,Pi) / Pred A_{max}(RuP_2-C)
		(μmoles m^{-2} s^{-1})				
E. crus-galli	+Na	59.5	52.3	209.3	4.00	1.14
	-Na	20.0	26.9	151.5	5.64	0.74
E. indica	+Na	74.0	63.6	290.6	4.57	1.16
	-Na	24.5	34.4	250.4	7.28	0.71
C. barbata	+Na	65.0	67.5	542.9	8.1	0.96
	-Na	22.0	32.9	551.6	16.8	0.67

[a] Maximum assimilation rates taken from CO_2 response curves shown in Figure 3.06a, b, c.

[b] The *in vitro* activities of RuP_2 carboxylase and PEP-carboxylase as expressed on a unit leaf area basis (see Section 5.3.1, Table 5.01).

or slightly greater than unity in control plants, they are consistently less than unity in sodium-deficient plants (see Table 3.03). Recognizing the capacities of PEP-carboxylase and RuP_2 carboxylase in sodium-deficient plants, and assuming the kinetic properties of these enzymes are unaffected by sodium nutrition, it seems unlikely that the carboxylation reactions, as such, are responsible for the apparent difference in observed and attainable rates of photosynthesis. Presumably some other reaction, or reactions, limit the overall photosynthetic capacity in these plants. Conceivably such limitations might also contribute towards the earlier saturation in the response of CO_2 assimilation to intercellular $p(CO_2)$ in sodium-deficient leaves.

Perhaps the increased PEP-carboxylase:RuP_2 carboxylase ratio of the sodium-deficient plant, as much as two-fold in the case of *C. barbata*, could perturb the stoichiometry between the C_4 acid carboxylase-decarboxylase system and the PCR cycle of the bundle-sheath compartment. In simple terms a relative increase in PEP-carboxylase capacity might induce an oversupply of C_4-acids and as a consequence there might be increased back-diffusion of CO_2 from the bundle-sheath compartment resulting in an inefficient and futile "recycling" of CO_2 by the C_4 pathway. Estimates of probable back-diffusion of CO_2 range from 10% (Hatch and Osmond 1976) up to 38% (Farquhar 1981). Farquhar (1981) discusses the relationship between carbon isotope discrimination and the intercellular $p(CO_2)$ in leaves and derives theoretical expressions relating to $\delta^{13}C$ values of leaves, the intercellular $p(CO_2)$, the physical discrimination by enzyme systems and the back-diffusion or "leakage" of CO_2 in C_4 plants. Table 3.04 shows theoretical estimates of back-diffusion or "leakage" of CO_2 in sodium-deficient and control plants of *E. crus-galli*, *E. indica* and *C. barbata*. These estimates were derived by substituting experimental

Table 3.04 A theoretical estimate of back-diffusion (ϕ) of CO_2 in sodium-deficient (-Na) and control plants(+Na) of *E. crus-galli*, *E. indica* and *C. barbata*.

Species and treatment		$\delta^{13}C_{plant}$ [a]	P_i [b]	ϕ [c]
E. crus-galli	+Na	-11.1	194	0.30
	-Na	- 8.0	278	0.19
E. indica	+Na	-12.1	159	0.36
	-Na	-10.5	193	0.27
C. barbata	+Na	-12.2	170	0.36
	-Na	-10.9	227	0.29

[a] Experimental $\delta^{13}C_{plant}$ (see Section 2.3.4, Table 2.07).

[b] Intercellular $p(CO_2)$ at ambient $p(CO_2)$ of 330 µbar (see Section 3.3.5, Figure 3.06a, b, c).

[c] ϕ is defined as the proportion of CO_2 produced by decarboxylation which leaks out of the bundle-sheath. This was calculated according to Farquhar (1981) where

$\delta_{plant} = \delta_{atm} - a - (b^* - a)\, P_i/P_a$ and

$b^* = b_4 + b_3\phi$ given:

(1) δ_{plant} is the $\delta^{13}C$ of plant material;

(2) δ_{atm} is the $\delta^{13}C$ of the atmosphere, i.e. ($-7^o/oo$);

(3) a is the fractionation associated with diffusion in air, i.e. ($4.4^o/oo$);

(4) b_4 is the effective discrimination against ^{13}C by PEP-carboxylase, i.e. ($-5.7^o/oo$);

(5) b_3 is the fractionation associated with RuP_2 carboxylase i.e. ($32^o/oo$);

(6) P_i and P_a are intercellular and external $p(CO_2)$ respectively.

values of the $\delta^{13}C$ (i.e. carbon isotope discrimination ratio, see Section 2.3. , Table 2.07), and the intercellular $p(CO_2)$ obtained at saturating light intensities and ambient CO_2 concentrations (i.e. 2 mE m^{-2} s^{-1}, 330 µbar CO_2, 21% O_2, see Section 3.3.5, Figure 3.06) into the theoretical equations of Farquhar (1981).

Importantly the bundle-sheath compartments of sodium-deficient plants would in effect be "tighter" as estimates of CO_2 "leakage" (i.e. ϕ, Table 3.04) in sodium-deficient plants are in every instance less than those obtained from control plants.

3.3.5 Stomatal conductance in sodium-deficient and control plants

Relationships between stomatal conductance to gaseous diffusion and irradiance, between stomatal conductance and intercellular $p(CO_2)$ and between the rate of CO_2 assimilation and stomatal conductance are shown for sodium-deficient and control plants of *E. crus-galli*, *E. indica* and *C. barbata*, and *A. edulis* and *A. nummularia* in Figures 3.08 and 3.09 respectively. The physiology of stomata in higher plants is complex (for reviews see Raschke 1975, Cowan 1977) and a detailed discussion of stomatal responses is beyond the scope of this study.

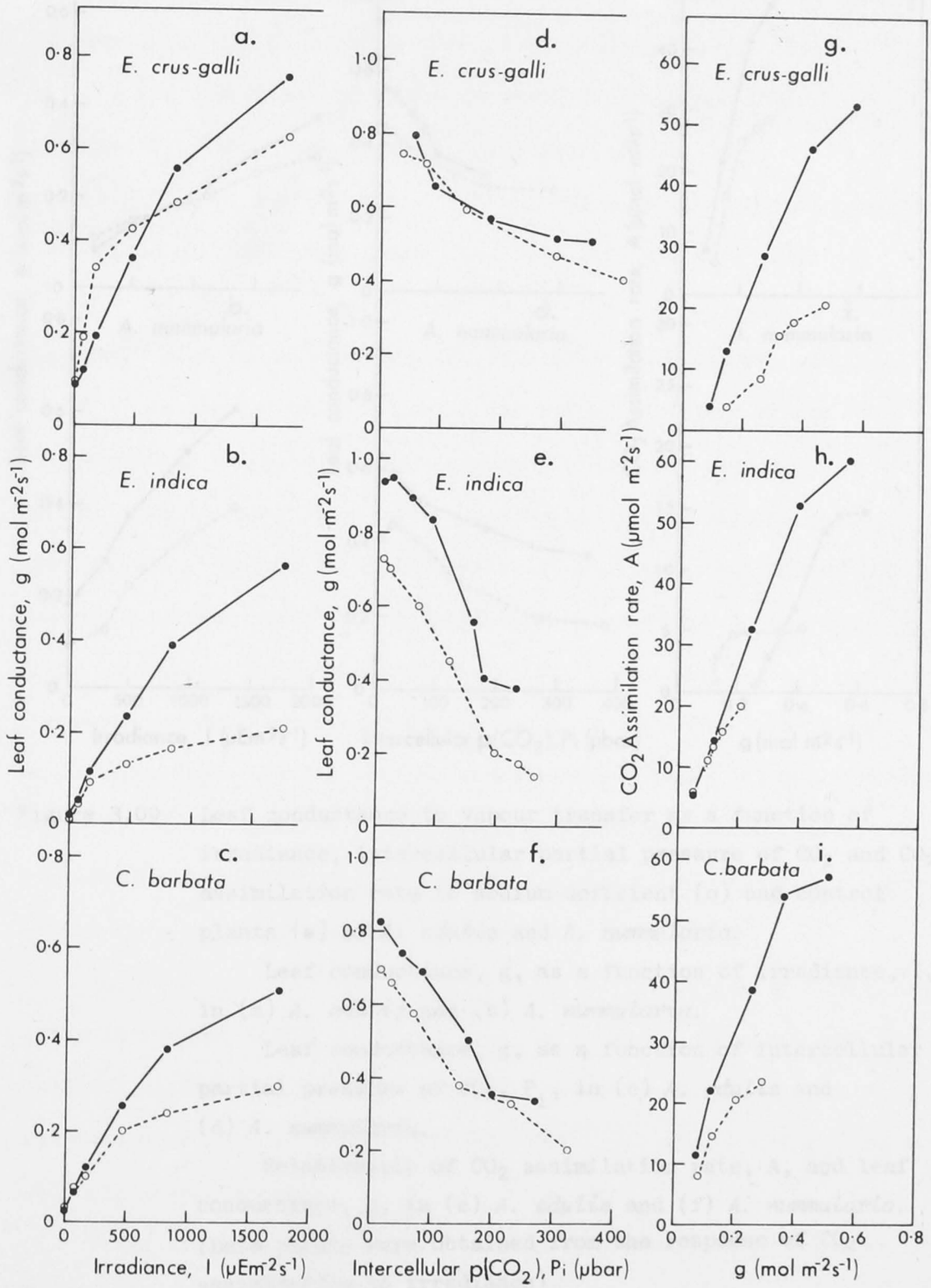
In the previous section I have shown that stomatal conductance is not limiting photosynthetic capacity in sodium-deficient plants (see Section 3.3.5). The higher intercellular $p(CO_2)$, obtained at ambient $p(CO_2)$ of 330 µbar, of sodium-deficient plants, is intriguing in the light of recent suggestions that stomatal conductance correlates with photosynthetic capacity (Wong 1979a, Wong *et al.* 1979). These authors observed that intercellular $p(CO_2)$ remained constant where the rate of CO_2 assimilation in *Zea mays* was altered through

Figure 3.08 Leaf conductance to vapour transfer as a function of irradiance, intercellular partial pressure of CO_2 and CO_2 assimilation rate in sodium-deficient (o) and control plants (•) of *E. crus-galli*, *E. indica* and *C. barbata*.

Leaf conductance, g, as a function of irradiance, I, in (a) *E. crus-galli*, (b) *E. indica* and (c) *C. barbata*.

Leaf conductance, g, as a function of intercellular partial pressure of CO_2, P_i, in (d) *E. crus-galli*, (e) *E. indica* and (f) *C. barbata*

Relationship of CO_2 assimilation rate, A, and leaf conductance, g, in (g) *E. crus-galli*, (h) *E. indica* and (i) *C. barbata*. (Data points were obtained from the response of CO_2 assimilation to irradiance.)


a.
E. crus-galli
b.
E. indica
c.
C. barbata
d.
E. crus-galli
e.
E. indica
f.
C. barbata
g.
E. crus-galli
h.
E. indica
i.
C.barbata
Leaf conductance, g (mol m-2s-1)
Leaf conductance, g (mol m-2s-1)
CO2 Assimilation rate, A (μmol m-2s-1)
Irradiance, I (μEm-2s-1)
Intercellular p(CO2), Pi (μbar)
g (mol m-2s-1)

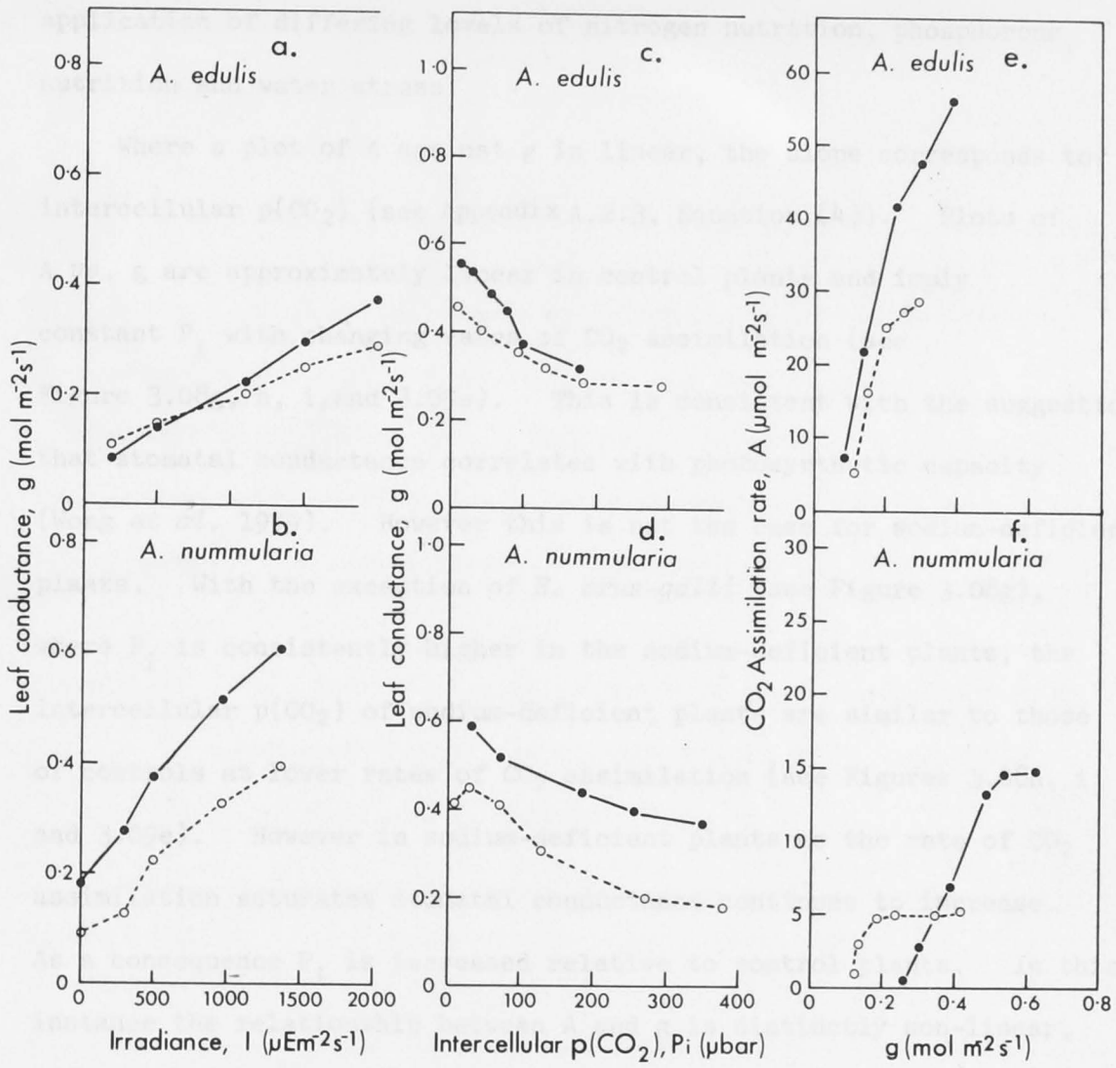


Figure 3.09 Leaf conductance to vapour transfer as a function of irradiance, intercellular partial pressure of CO_2 and CO_2 assimilation rate in sodium-deficient (o) and control plants (•) of *A. edulis* and *A. nummularia*.

Leaf conductance, g, as a function of irradiance, I, in (a) *A. edulis* and (b) *A. nummularia*.

Leaf conductance, g, as a function of intercellular partial pressure of CO_2, P_i, in (c) *A. edulis* and (d) *A. nummularia*.

Relationship of CO_2 assimilation rate, A, and leaf conductance, g, in (e) *A. edulis* and (f) *A. nummularia*. (Data points were obtained from the response of CO_2 assimilation to irradiance).

application of differing levels of nitrogen nutrition, phosphorous nutrition and water stress.

Where a plot of A against g is linear, the slope corresponds to intercellular $p(CO_2)$ (see Appendix A.2.3, Equation (4)). Plots of A *vs.* g are approximately linear in control plants and imply constant P_i with changing rates of CO_2 assimilation (see Figure 3.08g, h, i, and 3.09e). This is consistent with the suggestion that stomatal conductance correlates with photosynthetic capacity (Wong *et al.* 1979). However this is not the case for sodium-deficient plants. With the exception of *E. crus-galli* (see Figure 3.08g), where P_i is consistently higher in the sodium-deficient plants, the intercellular $p(CO_2)$ of sodium-deficient plants are similar to those of controls at lower rates of CO_2 assimilation (see Figures 3.08h, i, and 3.09e). However in sodium-deficient plants as the rate of CO_2 assimilation saturates stomatal conductance continues to increase. As a consequence P_i is increased relative to control plants. In this instance the relationship between A and g is distinctly non-linear, and a direct correlation between stomatal conductance and photosynthetic capacity is not apparent. Furthermore as photosynthetic capacity is reduced through sodium-deficiency, P_i does not remain constant. This is contrary to the presumption of Wong *et al.* 1979.

The responses of stomatal conductance to intercellular $p(CO_2)$ are shown in Figures 3.08d, e, f and 3.09c, d. While there are inter-specific differences in the response curves of stomatal conductance to intercellular $p(CO_2)$, those of sodium-deficient plants are similar to controls in all species.

While stomatal conductance in sodium-deficient plants might be of interest in relation to more general considerations of the interactions between stomatal physiology and mesophyll photosynthetic capacity,

within the scope of this study I conclude that stomatal conductance is not directly affected by sodium nutrition.

3.3.6 Sensitivity of photosynthesis to changing oxygen concentrations

Table 3.05 shows the effect of changing oxygen concentration on leaf photosynthesis in sodium-deficient and control plants of *E. crus-galli*, *E. indica* and *C. barbata*. In each of these species the light-saturated rate of photosynthesis is unaffected by either 2% O_2 or 50% O_2 where P_a is kept constant at 330 µbar.

Table 3.05 The effect of oxygen on the rate of CO_2 assimilation of sodium-deficient and control plants of *E. crus-galli*, *E. indica* and *C. barbata*.

Species and treatment		Rates of CO_2 Assimilation (µmol m^{-2} s^{-1})[a]		
		2% O_2	21% O_2	50% O_2
E. crus-galli	+Na	50	50	51
	-Na	23	21	23
E. indica	+Na	56	53	56
	-Na	21	20	18
C. barbata	+Na	50	48	49
	-Na	26	24	24

[a] Light intensity was 2 mE $m^{-2}s^{-1}$, P_a = 330 µbar, and leaf temperature was 28°C. Vapour pressure difference was 20 mbar.

Figure 3.10 shows the relationship between CO_2 assimilation rate and intercellular $p(CO_2)$ under 2% O_2, 21% O_2 and 50% O_2 in sodium-deficient and control plants of *C. barbata*.

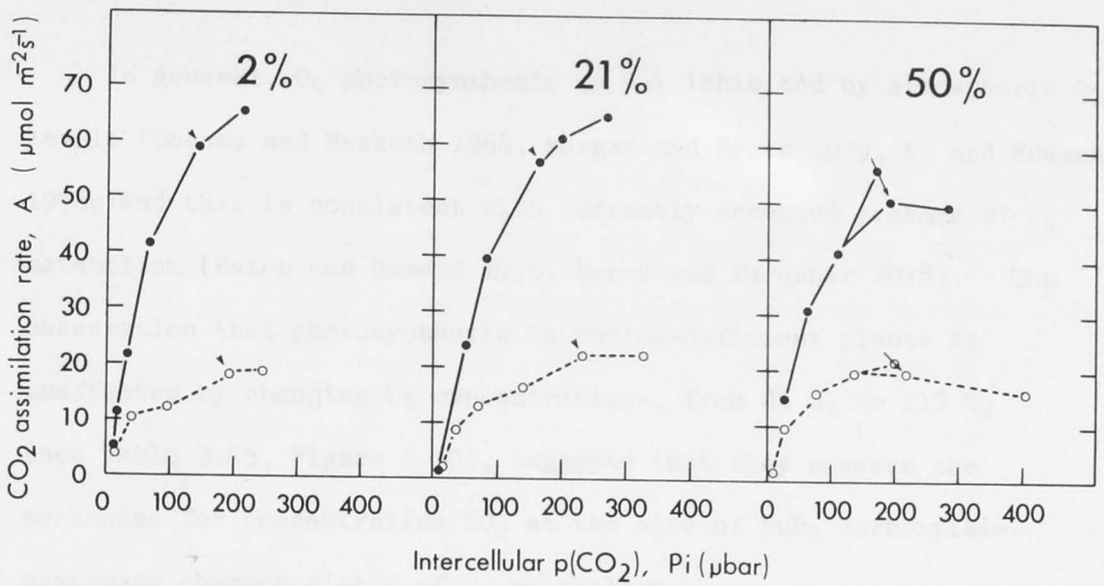


Figure 3.10 CO_2 response curves for sodium-deficient (o) and control plants (•) of *C. barbata* under 2% O_2, 21% O_2 and 50% O_2.

CO_2 assimilation rate, A, as a function of intercellular partial pressure of CO_2, P_i, under different oxygen regimes; 2% O_2, 21% O_2 and 50% O_2.

Leaf temperature was 28°C, vapour pressure difference was 20 mbar, and irradiance was 2 mE m^{-2} s^{-1}.

Values of A and p_i corresponding to P_a = 330 µbar are indicated by (◀).

There is no appreciable change in the CO_2 response curves, at low intercellular p(CO_2), under either 2% O_2, or 50% O_2, as compared to 21% O_2. However, there was a decrease in the rate of CO_2 assimilation after prolonged exposure to 50% O_2. After data points had been taken at low p(CO_2), photosynthesis did not return to the initial rates obtained at ambient p(CO_2). This decline was accompanied by a decrease in stomatal conductance and was not overcome by increasing external p(CO_2) in either sodium-deficient or control plants. As these observations were coincident with the end of the normal photoperiod, it was not possible to monitor any time-dependent reversal of this inhibition.

In general, C_4 photosynthesis is not inhibited by atmospheric O_2 levels (Downes and Hesketh 1968, Morgan and Brown 1979, Ku and Edwards 1978b)and this is consistent with currently accepted schemes of C_4 metabolism (Hatch and Osmond 1976, Berry and Farquhar 1978). The observation that photosynthesis in sodium-deficient plants is unaffected by changing O_2 concentrations, from 2% O_2 to 21% O_2 (see Table 3.05, Figure 3.10), suggests that they possess the mechanism for concentrating CO_2 at the site of RuP_2 carboxylase-oxygenase characteristic of C_4 metabolism.

Inhibition of C_4 photosynthesis by O_2 concentrations in excess of atmospheric levels would seem to vary between species and is largely dependent upon the time-course of experimental exposure (Forrester *et al.* 1966b, Gale and Tako 1976, Ku and Edwards 1980). In contrast to C_3 plants, where photosynthesis reaches steady-state within several minutes after changing O_2 concentration (Ku and Edwards 1980), several hours at very high O_2 concentration (70-100% O_2) is sometimes required to induce maximal inhibition in some C_4 plants (Gale and Tako 1976). In the species used in this study there was no appreciable change in photosynthesis after approximately 45 min exposure to 50% O_2 (see Table 3.05). Furthermore in *C. barbata* there was no evidence of inhibition at low intercellular $p(CO_2)$ under 50% O_2. Most importantly sodium-deficient plants were no more sensitive to 50% O_2 than were control plants. This, in conjunction with the observation that photosynthesis in sodium-deficient plants is unaffected by changing O_2 concentrations between 2% O_2 and 21% O_2 implies that C_4 pathway metabolism in these plants effectively suppresses photo-respiration.

3.3.7 Photosynthetic oxygen exchange

Figure 3.11 shows the components of photosynthetic O_2 exchange (i.e. gross O_2 production from water and gross O_2 uptake from the atmosphere) as a function of external $p(CO_2)$ in sodium-deficient and control plants of *E. indica* and *C. barbata*. These CO_2 response curves, as described by net O_2 evolution, show the same characteristics as those obtained in previous experiments where net CO_2 assimilation was measured as a function of intercellular $p(CO_2)$ (see Figures 3.06 and 3.07). In deficient and control plants of *E. indica* estimates of CO_2 uptake (calculated from the pressure *vs.* flow calibration of the CO_2 capillary inlet, see Appendix A.1) are given and are consistent with measured rates of net O_2 evolution.

Rates of O_2 uptake in both sodium-deficient and control plants are independent of CO_2 concentration and are very much lower than those observed in C_3 plants (Berry *et al.* 1978). Canvin *et al.* (1980) described similar rates of O_2 uptake in the C_4 plant, *A. edulis*. These authors suggest that photosynthetic oxygen uptake in C_4 plants is probably a combination of photorespiration and Mehler-type processes (i.e. direct photoreduction of O_2 supporting ATP synthesis *via* pseudo-cyclic photophosphorylation). However, relatively higher rates of O_2 uptake in sodium-deficient leaves of *C. barbata* might reflect increased rates of photorespiration in this plant.

Importantly, the low rates and CO_2-insensitivity of O_2 uptake seen in sodium-deficient plants imply that the direct fixation of atmospheric O_2 by RuP_2 carboxylase-oxygenase is effectively abolished. This is consistent with the observation that photosynthesis in sodium-deficient C_4 plants is unaffected by changing O_2 concentrations. There is therefore no evidence which would support the suggestion that sodium-deficiency

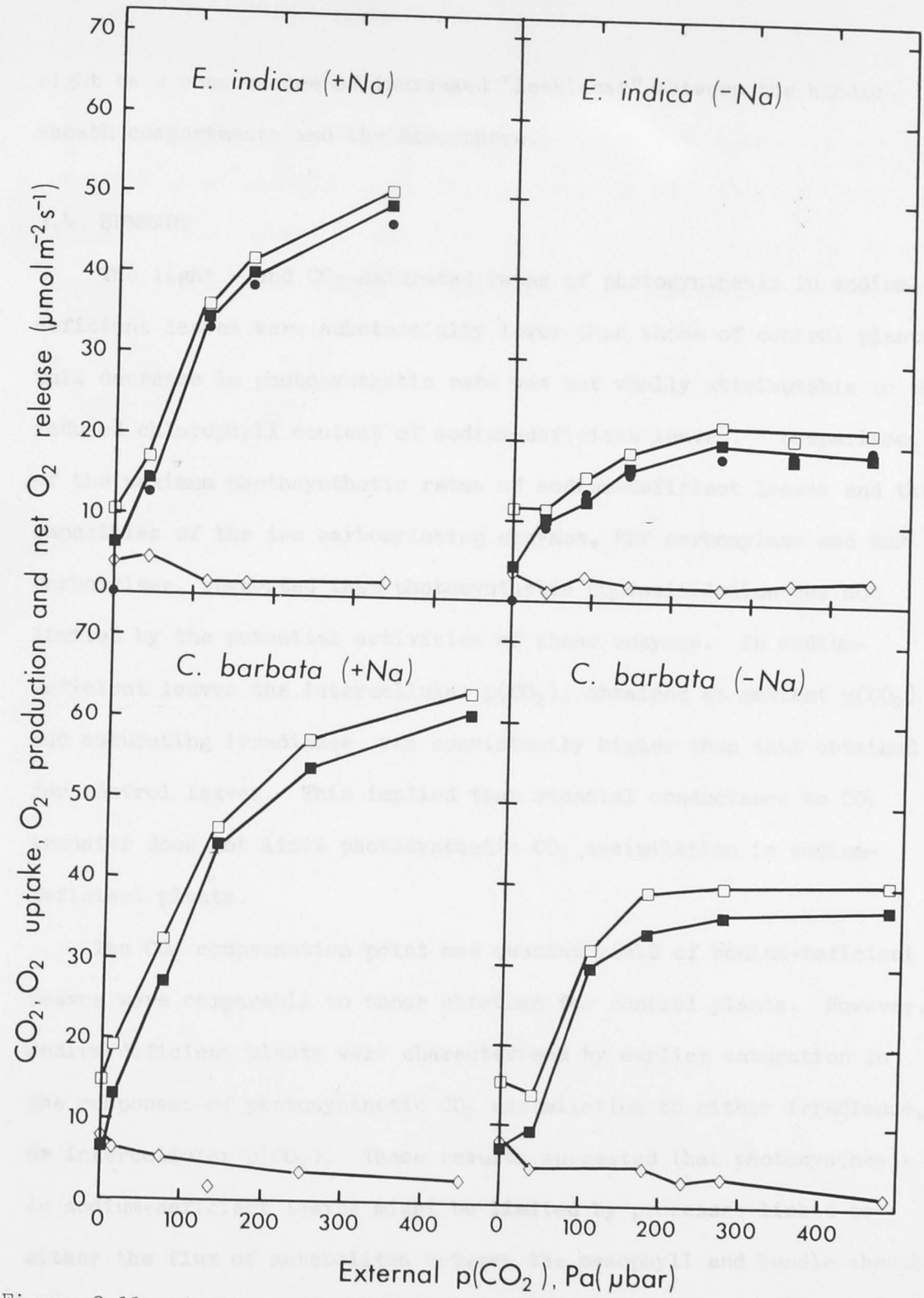


Figure 3.11 Oxygen exchange and CO_2 assimilation as a function of external partial pressure of CO_2, P_a, in sodium-deficient (-Na) and control plants (+Na) of *E. indica* and *C. barbata*. (●) CO_2 uptake, (◇) O_2 uptake, (□) O_2 production, (■) net O_2 release. Leaf temperature was 28°C and light intensity was 2 mE m^{-2} s^{-1}.

might be a consequence of increased "leakiness" between the bundle-sheath compartments and the atmosphere.

3.4 SUMMARY

The light - and CO_2-saturated rates of photosynthesis in sodium-deficient leaves were substantially lower than those of control plants. This decrease in photosynthetic rate was not wholly attributable to the reduced chlorophyll content of sodium-deficient leaves. Comparisons of the maximum photosynthetic rates of sodium-deficient leaves and the capacities of the two carboxylating enzymes, PEP carboxylase and RuP carboxylase, suggested that photosynthetic CO_2 assimilation was not limited by the potential activities of these enzymes. In sodium-deficient leaves the intercellular $p(CO_2)$, obtained at ambient $p(CO_2)$ and saturating irradiance, was consistently higher than that obtained for control leaves. This implied that stomatal conductance to CO_2 transfer does not limit photosynthetic CO_2 assimilation in sodium-deficient plants.

The CO_2 compensation point and quantum yield of sodium-deficient leaves were comparable to those obtained for control plants. However, sodium-deficient plants were characterised by earlier saturation in the responses of photosynthetic CO_2 assimilation to either irradiance, or intercellular $p(CO_2)$. These results suggested that photosynthesis in sodium-deficient leaves might be limited by processes linked to either the flux of metabolites between the mesophyll and bundle sheath compartments, or involved in the regenerative phases within these compartments.

The light saturated rate of photosynthesis in sodium-deficient leaves was unaffected by changing oxygen concentrations. Furthermore,

both sodium-deficient and control plants showed low rates of photosynthetic O_2 uptake which were CO_2-insensitive. These results confirmed that photosynthetic CO_2 assimilation *via* the C_4 pathway effectively abolishes direct fixation of atmospheric oxygen by RuP_2 oxygenase in both sodium-deficient and control leaves.

CHAPTER 4

RADIOTRACER KINETIC ANALYSIS OF C_4 PHOTOSYNTHESIS IN SODIUM-DEFICIENT PLANTS

4.1 INTRODUCTION

The studies reported in the previous chapter demonstrated that the physiological properties of gas exchange in intact leaves of sodium-deficient plants were consistent with C_4 photosynthetic metabolism. However, sodium-deficient plants were characterised by earlier saturation in the responses of CO_2 assimilation to either increasing irradiances, or increasing intercellular $p(CO_2)$. This suggests that reactions which limit the photosynthetic capacity of sodium-deficient leaves, while not apparent under low irradiance or low intercellular $p(CO_2)$, are exacerbated as the rate of photosynthetic CO_2 assimilation increases. I concluded that such limitations might be associated with either the flux of metabolites between the bundle-sheath and mesophyll compartments, or with processes involved in the regenerative phases within the bundle-sheath or mesophyll compartments.

In this chapter I will describe time-course and pulse-chase radiotracer studies of the incorporation of $^{14}CO_2$ into leaves of sodium-deficient and control plants during steady-state photosynthesis. These experiments provide more explicit information about component processes of C_4 photosynthesis in sodium-deficient leaves and are important in resolving the limiting process(es) associated with photosynthesis in these plants.

Analysis of radiotracer experiments has played an important role

in defining the component processes of C_4 photosynthesis (Kortschak *et al.* 1965, Hatch and Slack 1966, Hatch 1971). The general characteristics of radiotracer kinetics during steady-state C_4 photosynthesis have been the subject of several reviews (for example Hatch 1976a,b, Hatch and Osmond 1976) and are briefly outlined below.

During continuous feeding experiments in air labelled with $^{14}CO_2$, the C_4 acids malate, aspartate and oxaloacetate are rapidly labelled from zero time. With increasing time in $^{14}CO_2$, 3-PGA and other Calvin cycle intermediates enter a phase of rapid labelling which is followed by the accumulation of label in photosynthetic end products - sucrose and starch. Thus, the time-course labelling of C_4 acids gives a concave-negative slope which extrapolates to 100% of total incorporated (^{14}C) at zero time. Conversely, the time-course labelling of 3-PGA and its products show a convex-upwards slope extrapolating to approximately zero percent of incorporated (^{14}C) at zero time. These results imply that the C_4 acids are both initial products and primary intermediates of the C_4 pathway. The $^{14}CO_2$ entering C_4 acids is located in the C-4 position, while label entering 3-PGA appears in the C-1 position. During time-course experiments the C-4 of C_4 acids, and a relatively large internal pool of $^{14}CO_2$, saturate at about the same time, while saturation of the C-1 of 3-PGA is much slower. In pulse-chase experiments label is rapidly lost from the C-4 carboxyl of the C_4 acids, moves through 3-PGA and other intermediates and eventually appears in sugars and starch. The labelling kinetics of a free pool of $^{14}CO_2$ are consistent with it being intermediate between the C-4 of C_4 acids and the C-1 of 3-PGA. Interpretation of the labelling kinetics suggests that the path of carbon during C_4 photosynthesis can be summarised:-

External CO_2→C-4 of C_4 acids→intermediate CO_2 pool→C-1 of 3-PGA→products.

In the past there have been several (^{14}C) labelling studies with sodium-deficient C_4 plants. Unfortunately the experiments of Brownell and Osmond (unpublished results) and Holtum (1975) were not carried out under steady-state conditions and as a consequence the kinetic relationships were difficult to interpret. In this study two species were used in (^{14}C) labelling experiments. These were *Kochia childsii* Hort.,an NADP-ME type C_4 plant, and *Chloris barbata* Swartz, a PCK-type C_4 plant. Labelling experiments with *K.childsii* were done in collaboration with Mr. M. Webb at the James Cook University of North Queensland.

A survey of the free amino acid pool sizes in leaves of sodium-deficient plants is presented in the last section of this chapter. These are discussed in relation to the radiotracer kinetic characteristics of sodium-deficient leaves.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Sodium-deficient and control plants of *Amaranthus edulis Speg.*, *Atriplex inflata* Fv. M., *Kochia childsii* Hort., *Chloris barbata* Swartz., *Echinochloa crus-galli* var. *frumentaceae* (Roxb) and *Eleusine indica* (L) Gaertn. were obtained using the procedures for germination and growth of plants under low-sodium conditions described previously (see Sections 2.2.1 - 7). Plants of *K. childsii* and *C. barbata* used in the ^{14}C-labelling experiments were grown in a controlled environment chamber (see Section 2.2.2(B)). Plants of all species used in the determination of free amino acid pool sizes were grown in a naturally illuminated glasshouse under full sunlight (see Section 2.2.2(C)), with the exception of *K. childsii* which was grown as described above. At the time of

the experiments the age of the plants corresponded to those given previously (see Section 2.3.2, Table 2.02). In all experiments sodium-deficient and control plants were of the same age and only young fully expanded leaves were used.

4.2.2 Determination of short term photosynthetic products

Pulse-chase and continuous feeding experiments with $^{14}CO_2$ were conducted using the 3-litre exposure chamber described by Caldwell *et al.* (1977). The temperature of the chamber was controlled at 30°C in all experiments and two small high-speed fans within the chamber ensured rapid mixing of the air. The cuvette was illuminated with two 500W-incandescant lamps which irradiated the excised leaves at 900 μE m^{-2} s^{-1} (400-700 nm). Before each experiment the leaves were preconditioned in the light for 45 minutes and humidified air was passed through the chamber. The same air-stream was used during the chase period. Leaves were cut under water just prior to the labelling experiment and inserted into the chamber with the cut ends or petioles, covered with water-saturated cotton wool to prevent wilting in the course of the experiment. Experiments were carried out during the normal photoperiod and the chamber accommodated six leaf samples. In both sodium-deficient and control plants of *C. barbata* and *K. childsii* each leaf sample consisted of approximately 0.25 g fresh weight of young fully expanded leaves.

The $^{14}CO_2$ used in the labelling experiments was prepared in a round bottom flask to which mercury had been added to reduce the gaseous volume to approximately 50 ml. Following the addition of either 5 mCi of $NaH^{14}CO_3$ (Amersham Radiochemical, specific activity 58.5 mCi/mmol) or 5 mCi $Ba^{14}CO_3$ (Amersham Radiochemical, specific

activity 52.5 mCi/mmol), a Subaseal was inserted to keep the flask airtight, the flask was evacuated and an excess of 70% (v/v) perchloric acid was injected into the flask to liberate the $^{14}CO_2$. The flask was stored upside-down. When required, the necessary volume of $^{14}CO_2$ was removed using a syringe and an equivalent volume of mercury was injected into the flask to maintain constant pressure. For the labelling experiments with *K. childsii* and *C. barbata* approximately 0.5 mCi $^{14}CO_2$ was injected into the exposure chamber. The addition of $^{14}CO_2$ to the exposure chamber increased the total CO_2 concentration by less than 5%.

At pre-set times during the continuous feeding or pulse-chase experiments leaf samples were removed and killed immediately in boiling 80% (v/v) ethanol.

4.2.3 Tissue extraction and chromatographic procedures

After killing in 80% (v/v) ethanol samples were further extracted in 40% (v/v) ethanol and boiling water. Plant material was then removed, ground in a glass homogeniser, further extracted with boiling water, cooled and then recovered by centrifugation. The pellet was resuspended in water and an aliquot was counted in scintillation fluid. The bulk of the residue was filtered onto a pre-weighed glass-fibre disc and dried at 70°C to constant weight. The supernatant extracts were combined and pigments removed into a chloroform layer. The aqueous phase was made to volume and an aliquot counted in scintillation fluid. Aliquots taken from the chloroform phase were counted in scintillation fluid and used to estimate chlorophyll, after complete conversion to phaeophytin, according to Vernon (1960).

The aqueous extract was then separated into an amino acid fraction

by passage through Amberlite IR-120 (cation exchange resin), and another fraction containing carboxylic acids, phosphorylated compounds and neutral compounds. The amino acid fraction was recovered by elution with 10% (v/v) NH_4OH. The carboxylic acid, phosphorylated compounds and neutral compounds fraction was then separated into a carboxylic acids and phosphorylated compounds fraction, and a neutral compounds fraction by passage through Amberlite IRA-400 (anion exchange resin). The fraction containing carboxylic acids and phosphorylated compounds was recovered from the column by sequential elution with 1N and 4N formic acid. Aliquots of all fractions were counted in scintillation fluid and recovery of (^{14}C) was routinely better than 96%. The amino acid fraction, and the fraction containing carboxylic acids and phosphorylated compounds were dried under vacuum and aliquots from each were subjected to two-dimensional thin-layer chromatography.

An aliquot of the amino acid fraction was subjected to two-dimensional thin-layer chromatography using water-saturated phenol in the first, and propan-1-ol-H_2O-n-propyl acetate-acetic acid-pyridine (120:60:20:40:1,v/v) in the second dimension. Thin-layer plates were prepared by the method of Bieleski and Turner (1966).

The second fraction was subjected to two-dimensional thin-layer chromatography using ethanol-ammonia-water (4:1:1,v/v) in the first, and diethyl ether-formic acid (98% w/v) - water (7:2:1,v/v) in the second dimension. Thin-layer plates were prepared by the method of Schurmann (1969).

Labelled compounds located by autoradiography and identified by comparison with R_F-values previously obtained with standards were excised and counted in scintillation fluid. The (^{14}C) in each compound was expressed as a percentage of total (^{14}C) fixed.

The radioactivity of (^{14}C) in all samples was estimated using a Packard Tricarb 3330 Liquid Scintillation Spectrometer. The scintillation medium consisted of a fluor component (i.e. 1 litre of fluor contained 666 ml of toluene, 33 ml of Bridet Oil and 4 g of Omnifluor) and an aqueous component present in equal volumes.

4.2.4 Estimation of the free protein amino acid pools

Young fully-expanded leaves were excised, sealed in stoppered vials, and immediately frozen in liquid nitrogen prior to storage in a freezer at -20°C. Samples were then freeze-dried (-40°C in vacuo) to constant weight, and were then stored in a freezer at -20°C prior to tissue extraction and analysis. Each sample was equivalent to approximately 0.5 g fresh weight and samples were taken either in the middle of the normal photoperiod (designated as "light" samples) or 2 - 3 hours after the end of the photoperiod (designated as "dark" samples).

The dried leaf material was ground in a mortar and pestle with acid-washed sand and a small amount of liquid nitrogen. This was found to facilitate complete disintegration of the plant material. The ground leaf material was then extracted with 5 ml of 3% (w/v) sulphosalicylic acid (Yeoh and Chew, 1976). This homogenate was allowed to stand on ice for 30 min and was then centrifuged at (27,000 x g) for 30 min, at 0°C. The pellet was re-suspended in a further 5 ml of extraction medium, re-centrifuged for a further 20 min at 0°C and resultant supernatants pooled. After passing the sample through a column of Dowex-H^+ 50 w x 8(100 to 200 mesh) cation-exchange resin, the amino acids were eluted with 10% (v/v) NH_4OH and then evaporated to dryness on a rotary evaporator. The amino acid extract

was then taken up in 1.0 ml of 2N Na citrate pH 2.2 buffer and individual amino acids were determined quantitatively using a Beckman Amino Acid Analyser 119CL, programmed for the analysis of protein hydrolysates (Appendix A.3).

In these experiments arginine may be lost because it is unstable in 10% (v/v) NH_4OH, producing ornithine which will appear under the lysine peak in the subsequent analysis. Asparagine and glutamine were not determined, and in these analysies cochromatographed with serine and lysine. For these reasons, data for arginine, lysine and serine should be interpreted with caution.

In an experiment investigating the changes in free amino acid concentrations upon illumination, in sodium-deficient and control leaves of *K. childsii*, leaf samples were killed in 80% (v/v) ethanol and then extracted according to the method used in (^{14}C)labelling experiments (see Section 4.2.3). After elution from the cation-exchange column with 10% (v/v) NH_4OH, amino acids were determined quantitatively as outlined above.

4.3 RESULTS AND DISCUSSION

4.3.1 Radiotracer kinetic experiments

The incorporation of (^{14}C) into leaves of sodium-deficient and control plants, of both *K.childsii* and *C. barbata*, during the pulse-chase experiments, after 10 sec exposure to $^{14}CO_2$, and the time-course (i.e. steady state $^{14}CO_2$ fixation) experiments are shown in Figure 4.01. For all treatments the rate of $^{14}CO_2$ fixation throughout the time-course experiments were linear, suggesting that steady state conditions prevailed and that the total CO_2 concentration in the exposure chamber

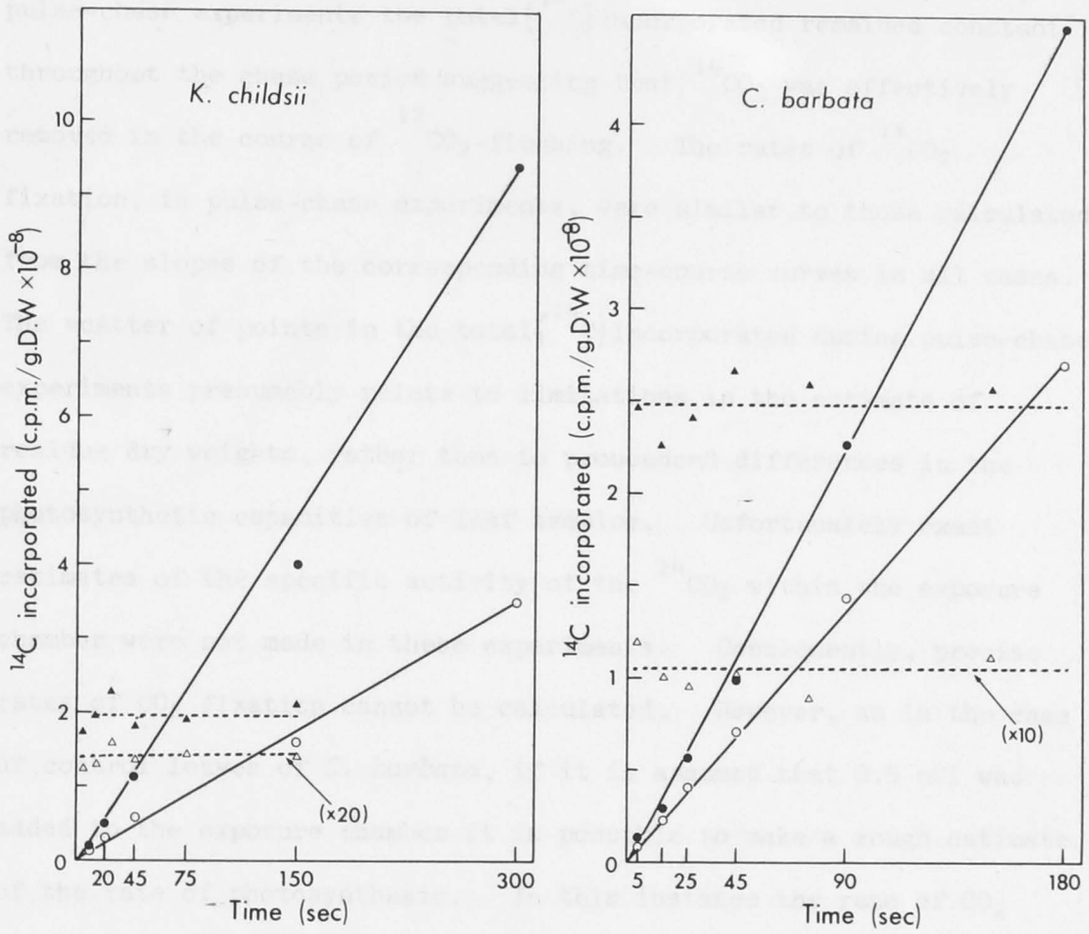


Figure 4.01 Time course of $^{14}CO_2$ incorporation into leaves of sodium-deficient and control plants of *K. childsii* and *C. barbata*. Total (^{14}C) incorporation is expressed as total c.p.m. incorporated g^{-1} dry wt of residue.

(●) control; steady state $^{14}CO_2$ fixation

(▲) control; pulse-chase experiment after 10-sec exposure to $^{14}CO_2$

(○) sodium-deficient; steady state $^{14}CO_2$ fixation

(△) sodium-deficient; pulse-chase experiment after 10-sec exposure to $^{14}CO_2$.

was not significantly depleted in any of these experiments. In the pulse-chase experiments the total (^{14}C) incorporated remained constant throughout the chase period suggesting that $^{14}CO_2$ was effectively removed in the course of $^{12}CO_2$-flushing. The rates of $^{14}CO_2$ fixation, in pulse-chase experiments, were similar to those calculated from the slopes of the corresponding time-course curves in all cases. The scatter of points in the total (^{14}C) incorporated during pulse-chase experiments presumably relate to limitations in the estimate of residue dry weights, rather than to pronounced differences in the photosynthetic capacities of leaf samples. Unfortunately exact estimates of the specific activity of the $^{14}CO_2$ within the exposure chamber were not made in these experiments. Consequently, precise rates of CO_2 fixation cannot be calculated. However, as in the case of control leaves of *C. barbata*, if it is assumed that 0.5 mCi was added to the exposure chamber it is possible to make a rough estimate of the rate of photosynthesis. In this instance the rate of CO_2 assimilation was found to be approximately 22 μmol CO_2 m^{-2} sec^{-1}. Given that these experiments were carried out at an irradiance less than half of full sunlight, and that the plants had been grown at relatively low irradiances (see Section 4.2.1) this rate is comparable to those measured previously (Chapter 3, Section 3.3.2-3). Importantly, the observed differences, in the rates of total (^{14}C) incorporation, between sodium-deficient and control leaves are consistent with the measured differences in the maximum photosynthetic capacity in these plants (see Chapter 3, Section 3.3.2-3). In the following discussion incorporation of (^{14}C) into the intermediates of the C_4 pathway are expressed as a percentage of the total (^{14}C) incorporated for all experiments.

The relationship between (^{14}C) label in the C_4 dicarboxylic acids (i.e. malate and aspartate) and the (^{14}C) label in 3-PGA and its products during the 10-sec $^{14}CO_2$ pulse, 150-sec $^{12}CO_2$ chase experiments, in the leaves of sodium-deficient and control plants of *K. childsii* and *C. barbata*, are shown in Figure 4.02. In this figure, the label in 3-PGA and its products is differentiated into total label in 3-PGA, phosphorylated compounds, sugars and insolubles, and total label in the free protein amino acids, alanine, glycine and serine. This is done in an attempt to highlight the major differences in the labelling kinetics of sodium-deficient and control leaves observed in these species.

Essentially, the labelling patterns of both sodium-deficient and control leaves of *K.childsii* and *C. barbata* are characteristic of C_4 photosynthesis. In all cases, there is a rapid initial loss of label from the C_4 dicarboxylic acids, which is accompanied by an increase in the label in 3-PGA and its products. This is consistent with product-precursor relationships in C_4 pathway metabolism (Hatch and Slack 1966, Hatch 1976a, b, Hatch and Osmond 1976). From Figure 4.02 it is clear that in sodium-deficient leaves, of both species, there is a much increased incorporation of label into the free amino acids, alanine, glycine and serine, as compared with that of control leaves. This label accumulates "at the expense of" sugars and insoluble materials. Furthermore, it is important to note that the accumulation of label in these amino acids is not due to direct CO_2 fixation, but that these compounds are produced following the primary fixation of CO_2 into the C_4 dicarboxylic acids. The significance of these differences, between sodium-deficient and control plants, are discussed in relation to more detailed pulse-chase, and time-course data presented in Figures 4.03 and 4.04.

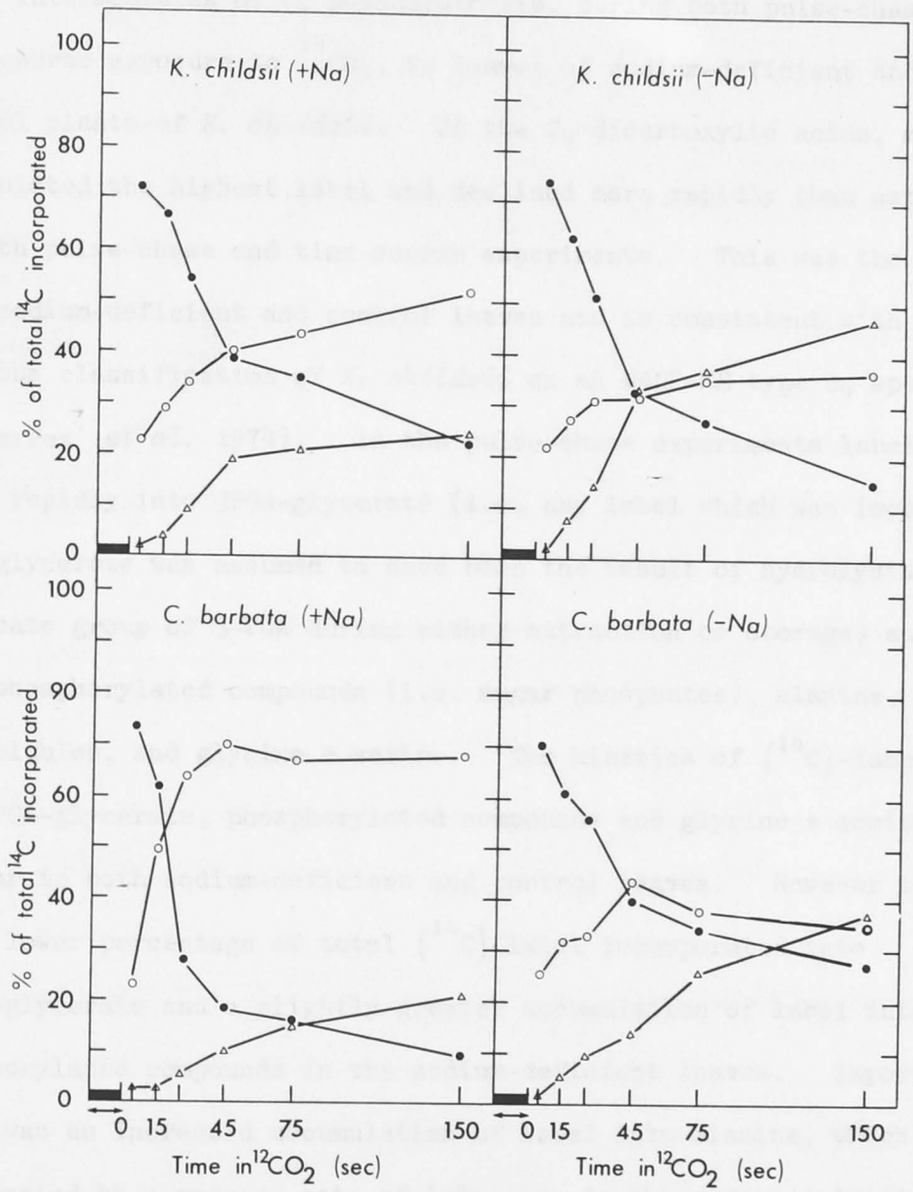


Figure 4.02 The relationship between (^{14}C) label in C_4 dicarboxylic acids (•), 3-PGA-glycerate and phosphorylated compounds and sugars + insoluble (o), and alanine and glycine + serine (Δ) after 10-sec exposure to $^{14}CO_2$ in sodium-deficient (-Na) and control leaves (+Na) of *K. childsii* and *C. barbata*.

Figure 4.03 describes the changes in the distribution of label among intermediates of C_4 photosynthesis, during both pulse-chase and time-course exposure to $^{14}CO_2$, in leaves of sodium-deficient and control plants of *K. childsii*. Of the C_4 dicarboxylic acids, malate accumulated the highest label and declined more rapidly than aspartate in both pulse-chase and time-course experiments. This was the case in both sodium-deficient and control leaves and is consistent with the previous classification of *K. childsii* as an NADP-ME type C_4 species (Gutierrez *et al.* 1974). In the pulse-chase experiments label moves rapidly into 3PGA-glycerate (i.e. any label which was incorporated into glycerate was assumed to have been the result of hydrolysis of the phosphate group of 3-PGA during either extraction or storage) and then into phosphorylated compounds (i.e. sugar phosphates), alanine, sugars + insolubles, and glycine + serine. The kinetics of (^{14}C)-labelling of 3-PGA-glycerate, phosphorylated compounds and glycine + serine are similar in both sodium-deficient and control leaves. However there was a lower percentage of total (^{14}C) label incorporated into 3-PGA-glycerate and a slightly greater accumulation of label into phosphorylated compounds in the sodium-deficient leaves. Importantly, there was an increased accumulation of label into alanine, which was accompanied by a reduced rate of labelling in the sugars + insoluble fraction, in the leaves of sodium-deficient plants. In the time-course experiment there was a slight decrease in the accumulation of label into 3-PGA-glycerate and glycine + serine, while there was a slight increase in the accumulation of label in phosphorylated compounds in the sodium-deficient leaves. The kinetics of the labelling in each of these intermediates were similar in both sodium-deficient and control leaves. The labelling of alanine and sugars + insolubles in the time-course experiments was consistent with the pulse-chase data. In the

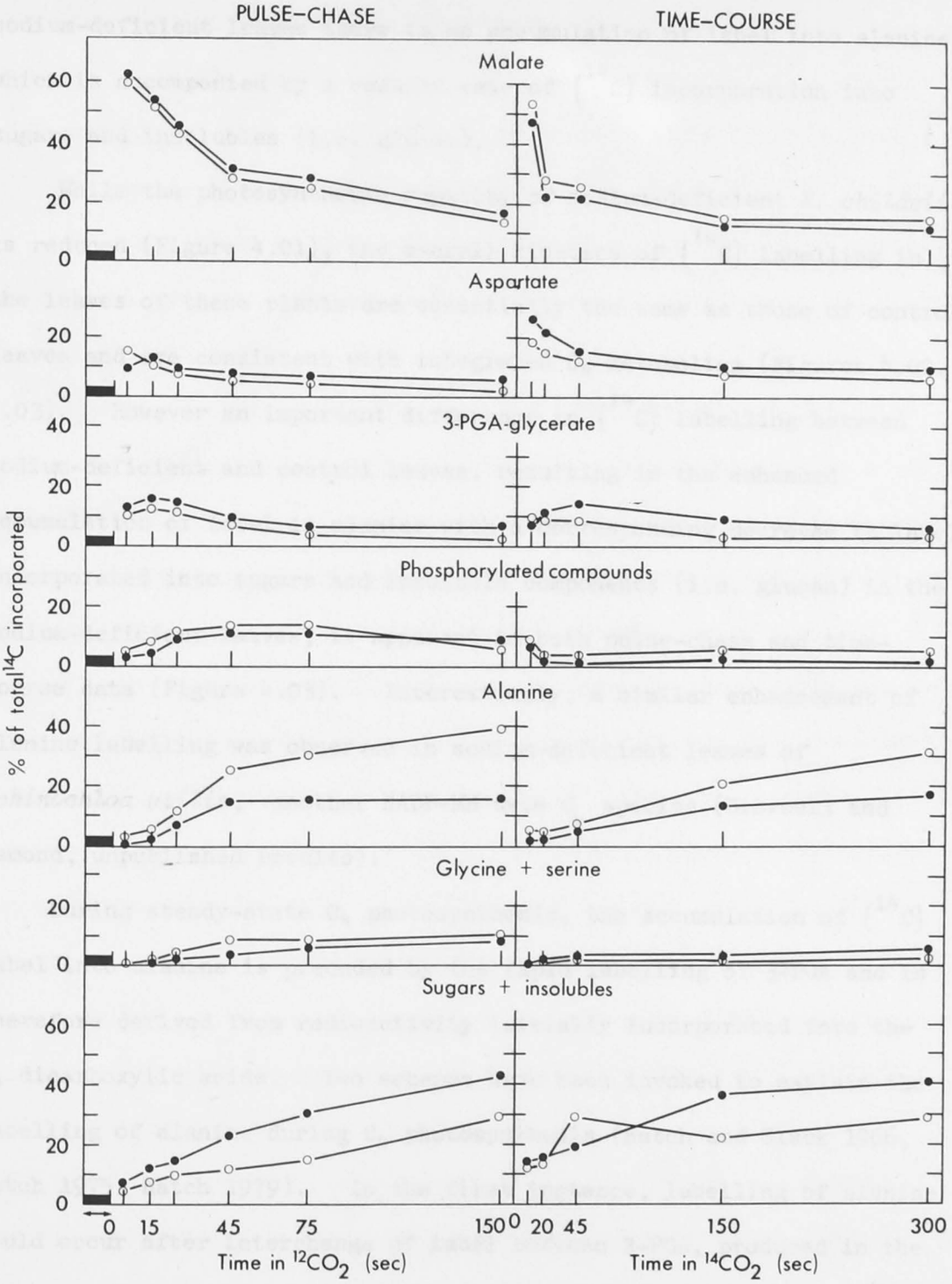


Figure 4.03 Changes in the distribution of label among intermediates of C_4 photosynthesis during pulse-chase and time-course exposure to $^{14}CO_2$ in leaves of sodium-deficient (o) and control plants (•) of *K. childsii*. Pulse-chase experiments were carried out after 10-sec exposure to $^{14}CO_2$.

sodium-deficient leaves there is an accumulation of label into alanine, which is accompanied by a reduced rate of (^{14}C) incorporation into sugars and insolubles (i.e. glucan).

While the photosynthetic capacity of sodium-deficient *K. childsii* is reduced (Figure 4.01), the overall kinetics of (^{14}C) labelling in the leaves of these plants are essentially the same as those of control leaves and are consistent with integrated C_4 metabolism (Figures 4.02, 4.03). However an important difference in (^{14}C) labelling between sodium-deficient and control leaves, resulting in the enhanced accumulation of label in alanine with a corresponding decrease in label incorporated into sugars and insoluble components (i.e. glucan) in the sodium-deficient leaves, is apparent in both pulse-chase and time-course data (Figure 4.03). Interestingly, a similar enhancement of alanine labelling was observed in sodium-deficient leaves of *Echinochloa utilis*; another NADP-ME type C_4 species (Brownell and Osmond, unpublished results).

During steady-state C_4 photosynthesis, the accumulation of (^{14}C) label into alanine is preceded by the rapid labelling of 3-PGA and is therefore derived from radioactivity initially incorporated into the C_4 dicarboxylic acids. Two schemes have been invoked to explain the labelling of alanine during C_4 photosynthesis (Hatch and Slack 1966, Hatch 1975, Hatch 1979). In the first instance, labelling of alanine would occur after interchange of label between 3-PGA, produced in the PCR-cycle, and PEP via reversible reactions catalysed by enolase and 3-PGA phosphomutase. After interchange of PEP and pyruvate, label could be transferred to alanine. Similarly, label could accumulate in alanine through the labelling of the C-1, C-2 and C-3 carbons of the dicarboxylic acids and, after decarboxylation, label could be transferred from pyruvate to alanine via alanine aminotransferase. It is in this

way that alanine labelling is thought to occur in NADP-ME type C_4 species. In the second scheme, label can be transferred to alanine following the C-4 to C-1 randomization of malate via fumarase in NAD-ME type C_4 species (Kagawa and Hatch 1974, Hatch 1975).

From the above it is likely that labelling of alanine is derived from label incorporated into 3-PGA in both sodium-deficient and control leaves of *K. childsii*. Given the various sources of 3-PGA, either produced in the bundle-sheath compartment and directly involved in PCR-cycle metabolism, or perhaps involved in the proposed flux of 3-PGA between bundle-sheath and mesophyll compartments (Hatch 1975, Hatch and Osmond 1976)and recognising that alanine aminotransferase probably occurs in both bundle-sheath and mesophyll compartments in these plants, I cannot define the specific location or mechanisms responsible for the observed labelling of alanine. Recognising that the amount of label appearing in photosynthetic intermediates is partly a function of their pool size, the enhanced accumulation of label in alanine observed in sodium-deficient leaves of *K.childsii* might simply reflect relative differences in the magnitude of either the 3-PGA, or alanine pools of these leaves. Consequently, a reduced 3-PGA pool size or an increased alanine pool size would contribute towards increased labelling of alanine in sodium-deficient plants.

The data presented in Figure 4.03 do not provide an estimate of the 3-PGA pool size as the total counts incorporated into 3-PGA did not saturate within the time-course of the experiment. However, movement of label through 3-PGA and into subsequent intermediates is essentially the same for sodium-deficient and control leaves and would suggest that, in relation to the overall photosynthetic capacity of sodium-deficient and control leaves, the pool sizes of 3-PGA are essentially similar. Data presented in a following section (see Section 4.3.2) shows that

the total leaf pool of alanine is significantly increased in sodium-deficient leaves of *K. childsii* and in several other C_4 species. In addition, alanine aminotransferase activities, in whole leaf extracts, of sodium-deficient leaves of *E. crus-galli*, *E. indica* and *C. barbata* are relatively higher than those of control leaves (see Chapter 5, Section 5.3.1).

Figure 4.04 describes the changes in the distribution of label among intermediates of C_4 photosynthesis, during both pulse-chase and time-course exposure to $^{14}CO_2$, in leaves of sodium-deficient and control plants of *C. barbata*. In both pulse-chase and time-course experiments malate, and not aspartate, would appear to be the major C_4 dicarboxylic acid involved in the transfer of carbon to the bundle-sheath compartment in the leaves of sodium-deficient plants. In the pulse-chase experiments the label in aspartate is lost quite rapidly in control leaves, while that in malate accumulates in the early chase period and then rapidly declines. However in the sodium-deficient leaves, label in the C_4 acids declines more slowly (see also Figure 4.01) in both malate and aspartate. During the time-course experiments label in aspartate declines rapidly, and remains at a low and steady level in malate in control leaves. In the time-course study with sodium-deficient leaves of *C. barbata* label is fairly evenly distributed between malate and aspartate and declines at a lower rate than the controls. *C. barbata* possesses high activities of PEP-carboxykinase (see Chapter 5, Section 5.3.1) and possesses ultrastructural features (see Chapter 6, Section 6.3.1) characteristic of a PCK-type C_4 species. The higher proportion of labelling in aspartate, seen in the control leaves, is consistent with these observations. The labelling kinetics of 3-PGA and alanine are similar for sodium-deficient and control leaves in both pulse-chase and time-course experiments. However in

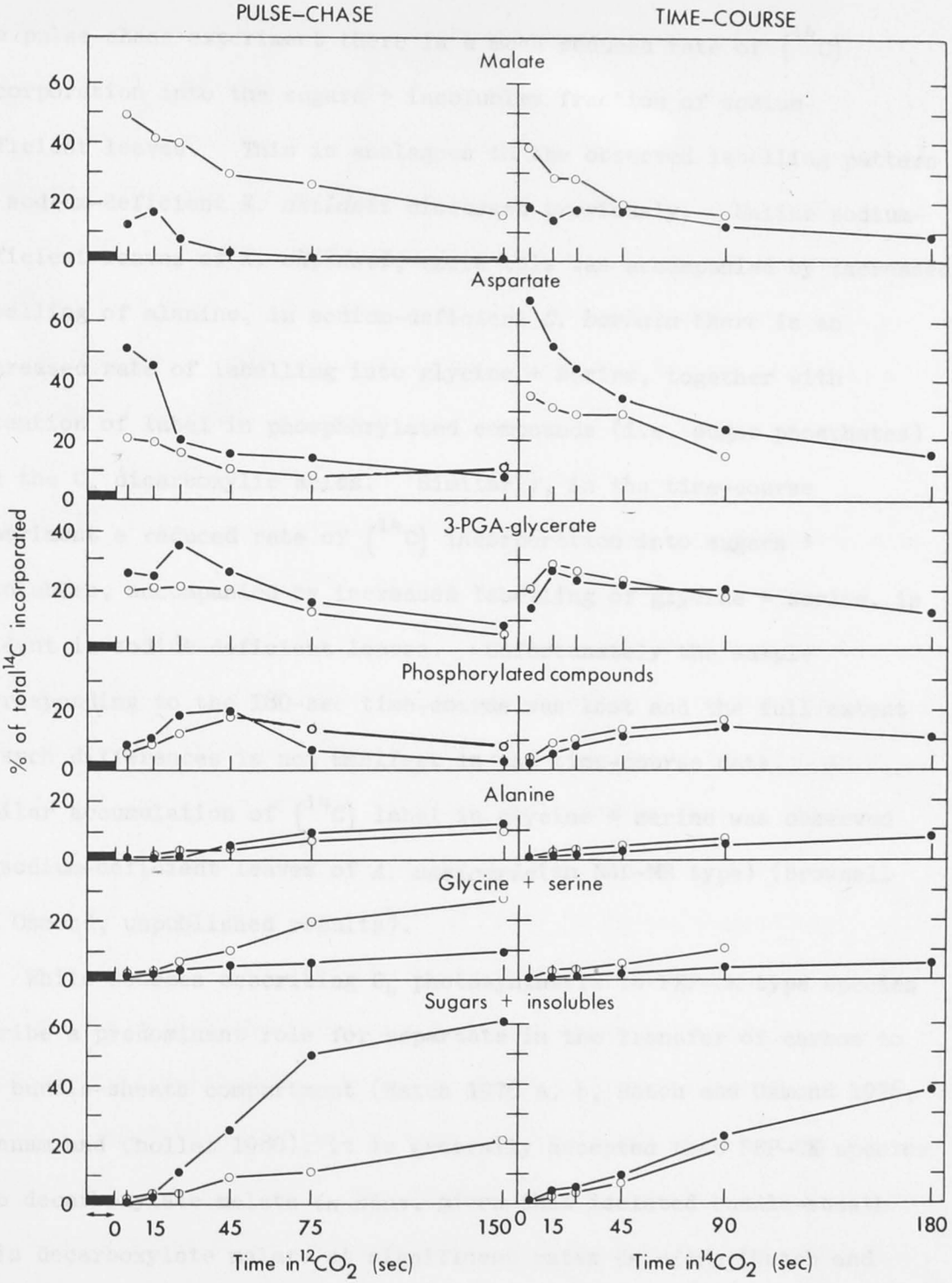


Figure 4.04 Changes in the distribution of label among intermediates of C_4 photosynthesis during pulse-chase and time-course exposure to $^{14}CO_2$ in leaves of sodium-deficient (o) and control plants (•) of *C. barbata*. Pulse-chase experiments were carried out after 10-sec exposure to $^{14}CO_2$.

the pulse-chase experiment there is a much reduced rate of (^{14}C) incorporation into the sugars + insolubles fraction of sodium-deficient leaves. This is analagous to the observed labelling pattern in sodium-deficient *K. childsii* discussed previously. Unlike sodium-deficient leaves of *K. childsii*, where this was accompanied by increased labelling of alanine, in sodium-deficient *C. barbata* there is an increased rate of labelling into glycine + serine, together with retention of label in phosphorylated compounds (i.e. sugar phosphates) and the C_4 dicarboxylic acids. Similarly, in the time-course experiment a reduced rate of (^{14}C) incorporation into sugars + insolubles, accompanied by increased labelling of glycine + serine, is evident in sodium-deficient leaves. Unfortunately the sample corresponding to the 180-sec time-course was lost and the full extent of such differences is not manifest in the time-course data. A similar accumulation of (^{14}C) label in glycine + serine was observed in sodium-deficient leaves of *A. vesicaria* (an NAD-ME type) (Brownell and Osmond, unpublished results).

While schemes describing C_4 photosynthesis in PEP-CK type species ascribe a predominant role for aspartate in the transfer of carbon to the bundle-sheath compartment (Hatch 1976 a, b, Hatch and Osmond 1976, Rathnam and Chollet 1980), it is generally accepted that PEP-CK species also decarboxylate malate *in vivo*, given that isolated bundle-sheath cells decarboxylate malate at significant rates *in vitro* (Hatch and Kagawa 1976, Rathnam and Edwards 1977). The increased labelling of malate seen in pulse-chase and time-course experiments with sodium-deficient leaves of *C. barbata* could suggest that these plants possess a relatively greater capacity for malate decarboxylation in the bundle-sheath cells. Anticipating results presented in the following chapter (see Section 5.3.1) the levels of NAD -malate dehydrogenase and

aspartate aminotransferase in sodium-deficient leaves of *C. barbata* are increased relative to controls. Higher activities of these enzymes might facilitate either enhanced malate decarboxylation or rapid equilibration between malate and aspartate via oxaloacetate. Furthermore, the total leaf aspartate pool in sodium-deficient plants of *C. barbata* is significantly lower than that of control leaves (see Section 4.3.2). This could contribute towards a change in relative labelling of aspartate and malate if the pool sizes in the plants used in these radiotracer experiments are comparable.

(^{14}C) labelling of glycine and serine during C_4 photosynthesis is much lower than that observed during C_3 photosynthesis (Osmond and Harris 1971, Osmond and Björkman 1972). While this may be attributable to relatively low capacities of glycolate pathway enzymes in the leaves of C_4 species (Osmond and Harris 1971), this reduced flow of carbon through the glycolate pathway is primarily a consequence of the high CO_2:O_2 ratio which prevails in the bundle-sheath compartment during steady-state C_4 photosynthesis (Hatch 1971, Hatch and Osmond 1976, Canvin 1979). This high CO_2:O_2 ratio suppresses the oxygenation of RuP_2 by RuP_2 carboxylase-oxygenase and diminishes the production of phosphoglycolate and glycolate; precursors of glycine and serine within the glycolate pathway (Tolbert and Ryan 1974, 1976). Perturbation of the CO_2:O_2 ratio, within the bundle-sheath compartment has been shown to influence the flux of carbon through the glycolate pathway during C_4 photosynthesis. For example, increasing the atmospheric O_2 concentration, from 2% O_2 to 21% O_2, stimulates the labelling of glycolate pathway intermediates during C_4 photosynthesis (Osmond and Björkman 1972, Lawlor and Fock 1978, Morot-Gaudry *et al.* 1980). In $^{14}CO_2$ pulse-chase experiments, where CO_2-free air is used throughout the chase period, labelling of glycolate pathway intermediates (i.e. glycine + serine)

increased, as the internal CO_2 pool was depleted, and RuP_2 oxygenation was stimulated (Morot-Gaudry *et al.* 1980). In an analogous manner, labelling of glycolate pathway intermediates increased as a result of rapidly induced water stress in maize (Lawlor and Fock 1978). In this instance, perturbation of the internal CO_2:O_2 ratio was due to the sharp decline in stomatal conductance as a result of water stress. This restricted CO_2 uptake from the atmosphere, limited the dicarboxylic acid flux between the mesophyll and bundle-sheath compartments and by reducing the internal CO_2 concentration resulted in enhanced RuP_2 oxygenation and subsequent glycolate metabolism.

From (^{14}C) labelling experiments with C_4 plants it is evident that metabolism of glycine is not stoichiometrically linked to serine production (Osmond and Björkman 1972, Lawlor and Fock 1978, Morot-Gaudry *et al.* 1980). In effect, part of the (^{14}C) serine pool is presumably derived from an intermediate other than (^{14}C) glycine during C_4 photosynthesis. It is suggested that the most likely route for serine synthesis, other than via the glycolate pathway, is from 3-PGA via glycerate and 3-hydroxypyruvate in reactions catalysed by 3-PGA phosphatase and D-glycerate dehydrogenase (Randall *et al.* 1971, Lawlor and Fock 1978, Morot-Gaudry *et al.* 1980).

From the above, increased labelling of glycine + serine in sodium-deficient *C. barbata* suggests that there might be enhanced RuP_2-oxygenase activity within the bundle-sheath compartment of these leaves. However, from the gas exchange data presented in the previous chapter (see Chapter 3, Sections 3.3.5 - 6), sodium-deficient leaves of *C. barbata* are no more sensitive to changing atmospheric oxygen concentrations than control leaves. In addition, low rates of O_2 uptake, under normal atmospheric O_2 and CO_2 concentrations, do not suggest substantial fixation of atmospheric O_2 via RuP_2 oxygenase in these leaves.

As the distribution of label between glycine and serine was not estimated in these experiments I cannot assess the magnitude of serine labelling *via* the non-glycolate pathway route (i.e. from 3-PGA *via* glycerate and 3-hydroxypyruvate) in either sodium-deficient or control leaves of *C. barbata*.

In sodium-deficient leaves label might also accumulate in glycine + serine if the pool sizes of these intermediates were greater than those of the controls. Data presented in the following section (see Section 4.3.2) support this interpretation. However, plants of *C. barbata* which were used for the determination of free amino acid pool sizes were grown under a different growth regime from those used in these labelling experiments (see Section 4.2.1). As a consequence, direct comparisons cannot be made in this instance.

4.3.2 The concentration of free amino acids in leaves of sodium-deficient and control plants

Observed increases in (^{14}C)-labelling of either alanine in sodium-deficient leaves of *K. childsii* and *E. utilis* (Brownell and Osmond, unpublished results), or glycine + serine in sodium-deficient leaves of *C. barbata* and *A. vesicaria* (Brownell and Osmond, unpublished results), prompted me to examine the free amino acid pool sizes in the leaves of sodium-deficient and control plants of several C_4 species.

The concentrations of free amino acids in leaves of sodium-deficient and control plants of *K. childsii*, *A. inflata* and *A. edulis*, and *E. crus-galli*, *E. indica* and *C. barbata* are shown in Tables 4.01 and 4.02, respectively. With the exception of *C. barbata*, the total free amino acid concentration in leaves of sodium-deficient plants were

Table 4.01 The concentrations of free amino acids (μmol g^{-1} dry wt) in leaves of sodium-deficient and control plants of *K. childsii*, *A. inflata* and *A. edulis*. Each value is the mean of three replicates and the significance (Students' t-test) between the sodium-deficient and control plants is indicated by: a = P 0.001; b = P 0.01; c = P 0.05. (+Na) control; (-Na) sodium-deficient plant.

Amino Acid	*K. childsii*		*A. inflata*		*A. edulis*	
	+Na	-Na	+Na	-Na	+Na	-Na
Asp	2.2	21.2[b]	9.0	4.1	7.6	4.1
Thr	0.9	<0.1	<0.1	2.8[a]	<0.1	<0.1
Ser	21.5	48.9[b]	30.8	20.2	35.5	47.7[c]
Glu	13.3	97.8[a]	44.7	56.6[c]	48.0	87.3[a]
Pro	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Gly	31.1	34.5	6.8	7.5	2.3	31.0[b]
Ala	4.0	140.4[a]	36.1	61.5[c]	17.9	15.9
Cys	<0.1	0.9[c]	<0.1	<0.1	<0.1	<0.1
Val	1.3	2.3[b]	4.7	0.8[a]	0.2	<0.1[c]
Met	<0.1	0.2	0.1	<0.1[b]	0.3	1.2[b]
Ile	1.0	2.0[a]	3.1	1.0[a]	0.6	0.2
Leu	1.8	2.7[c]	2.3	0.9[a]	0.6	0.5
Tyr	0.3	0.7[a]	0.7	0.2[b]	0.2	<0.1[b]
Phe	0.4	1.6[a]	0.9	0.8	0.7	1.1[a]
His	1.2	1.3	3.7	2.4[c]	0.2	0.4[b]
Lys	2.9	0.8[c]	4.8	1.8[b]	0.3	0.4
Arg	5.9	<0.1[a]	17.4	60.2[a]	0.3	<0.1
TOTAL	88.4	355.5[a]	164.8	220.4[c]	114.3	189.7[b]

Table 4.02 The concentrations of free amino acids (μmol g^{-1} dry wt) in leaves of sodium-deficient and control plants of *E. crus-galli*, *E. indica* and *C. barbata*. Each value is the mean of three replicates and the significance (Students' t-test) between the sodium-deficient and control plants is indicated by: a = P 0.001; b = P 0.01; c = P 0.05. (+Na) control; (-Na) sodium-deficient plant.

Amino Acid	*E. crus-galli*		*E. indica*		*C. barbata*	
	+Na	-Na	+Na	-Na	+Na	-Na
Asp	4.5	8.4	28.2	9.4[b]	21.5	7.1[a]
Thr	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Ser	11.2	58.5[b]	64.3	57.3	111.6	97.6
Glu	6.6	39.2[a]	61.3	74.8	65.0	47.9[b]
Pro	0.3	<0.1	2.2	2.0	27.1	12.3[c]
Gly	3.4	30.6[c]	14.6	44.5[b]	29.0	34.5
Ala	10.0	220.8[a]	48.8	149.4[a]	121.3	174.6[b]
Cys	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Val	0.7	3.6	2.0	3.9	2.4	4.2[a]
Met	0.1	0.4	0.6	1.6[c]	1.4	1.9
Ile	0.2	1.4[b]	1.1	1.5	1.3	1.7
Leu	0.3	1.2	1.6	1.8	1.6	3.3
Tyr	0.2	0.6	0.4	0.4	<0.1	0.1
Phe	0.4	1.5	0.6	0.9	0.9	10.5
His	0.4	0.7	0.8	0.7	1.2	1.9[c]
Lys	0.5	0.9	1.2	0.8	1.3	1.7
Arg	<0.1	0.2	1.2	1.1	<0.1	0.2
TOTAL	38.1	363.9[a]	226.5	348.8[b]	388.9	399.3

significantly greater than those of control plants in all of the species examined. The amino acids making the major contribution to these differences were; alanine, glycine, glutamic acid, serine and aspartic acid. However, there was considerable interspecific variation in the relative contribution of these individual amino acids to the overall increases in total free amino acid content of sodium-deficient leaves.

With the exception of *A. edulis*, sodium-deficiency resulted in a significant accumulation of alanine in each of the species examined. In sodium-deficient leaves of *K. childsii* and *E. crus-galli* the concentrations of alanine were greater than twenty-fold those of control leaves. In *E. crus-galli*, *E. indica* and *A. edulis* the concentrations of glycine in the leaves of sodium-deficient plants were significantly greater than those of control plants. In sodium-deficient leaves of *E. crus-galli* and *A. edulis* the concentrations of glycine were greater than ten-fold those of control leaves. However, there was no significant change in the concentration of glycine in *K. childsii*, *C. barbata* and *A. inflata* as a result of sodium-deficiency. The concentrations of glutamic acid in sodium-deficient leaves of *K. childsii*, *A. inflata*, *A. edulis* and *E. crus-galli* were significantly greater than those of control plants. However the concentration of glutamic acid in leaves of *C. barbata* decreased, while that of *E. indica* was unaffected, as a result of sodium-deficiency. The serine concentration increased significantly in sodium-deficient leaves of *E. crus-galli*, *K. childsii* and *A. edulis*, but remained constant in the other species examined. The concentration of aspartic acid decreased in *C. barbata* and *E. indica*, was unaltered in *E. crus-galli*, *A. inflata* and *A. edulis*, and increased in *K. childsii* as a result of sodium-deficiency. Additionally, the concentration of arginine increased significantly in *A. inflata*, while it decreased in *K. childsii*

as a result of sodium-deficiency.

While high free amino acid concentrations are characteristic of sodium-deficient plants, there is considerable interspecific variation in the relative contributions of individual amino acids. Although the largest differences between sodium-deficient and control plants with respect to alanine accumulation were observed in the two NADP-ME type species, *E.crus-galli* and *K. childsii*, there does not appear to be any consistent pattern in the composition of the total free amino acid pools of malate-forming species as compared to aspartate formers.

In general, many of the free protein amino acids show increases or decreases in response to mineral deficiencies, and there is considerable interspecific variation in the relative composition of accumulated soluble nitrogen, including amino acids, with respect to specific mineral deficiencies (Possingham 1956, Steward *et al.* 1959, Stewart and Larher 1980). However the high concentrations of free protein amino acids observed in sodium-deficient leaves might be directly related to C_4 photosynthetic metabolism in these plants. For example, an accumulation of alanine in sodium-deficient leaves could, in simple terms, reflect a disfunction in the processes associated with the conversion of pyruvate to phosphoenolpyruvate in the mesophyll chloroplasts. Alternatively, the accummulation of large concentrations of free amino acids could affect the flux of metabolites between bundle-sheath and mesophyll compartment through perturbation of concentration gradients. It is difficult to qualify these possibilities with reference to the total free protein amino acid contents presented in Tables 4.01 and 4.02. Clearly, such measurements do not differentiate either between photosynthetic and non-photosynthetic pools, or, in the case of C_4 plants, between component pools of the mesophyll and bundle-

sheath compartments. In an attempt to differentiate between photosynthetic and non-photosynthetic pools two strategies were used.

In the first instance, excised leaves of sodium-deficient and control plants of *K.childsii* were preconditioned in the dark, for 45 minutes, in the exposure chamber used for radiotracer studies. Following dark equilibration, leaves were irradiated (i.e. 1 mE m^{-2} s^{-1}) and samples killed at pre-set intervals throughout the light-chase period for determination of amino acid content. The relative changes in the total leaf concentrations of alanine, glycine, glutamic acid, serine and aspartic acid in sodium-deficient and control leaves of *K. childsii* upon illumination are shown in Figure 4.05. It is evident that the absolute concentrations of some free protein amino acids change rapidly throughout this dark-light transition. Absolute changes in the pool sizes of free amino acids and photosynthetic intermediates during dark-light transitions have been reported for other C_4 species (Farineau 1971, Hatch 1979). In control leaves of *K. childsii*, the concentrations of serine, alanine, and to a lesser extent aspartic acid, decrease upon illumination. The concentration of glycine increases while that of glutamic acid does not change. In sodium-deficient leaves of *K. childsii*, the concentration of glycine increases while those of aspartic acid and serine decrease upon illumination. The concentration of alanine exhibits a transient decrease while that of glutamic acid does not change upon illumination.

Tentative interpretation of this data suggests that a substantial part of the glycine pool is directly related to photosynthetic metabolism in the leaves of both sodium-deficient and control plants. Concomitent decreases in the absolute concentrations of serine suggest that this pool is involved as an amino donor via serine hydroxymethyl transferase (Tolbert 1971, Keys 1980). The observed decline in the absolute

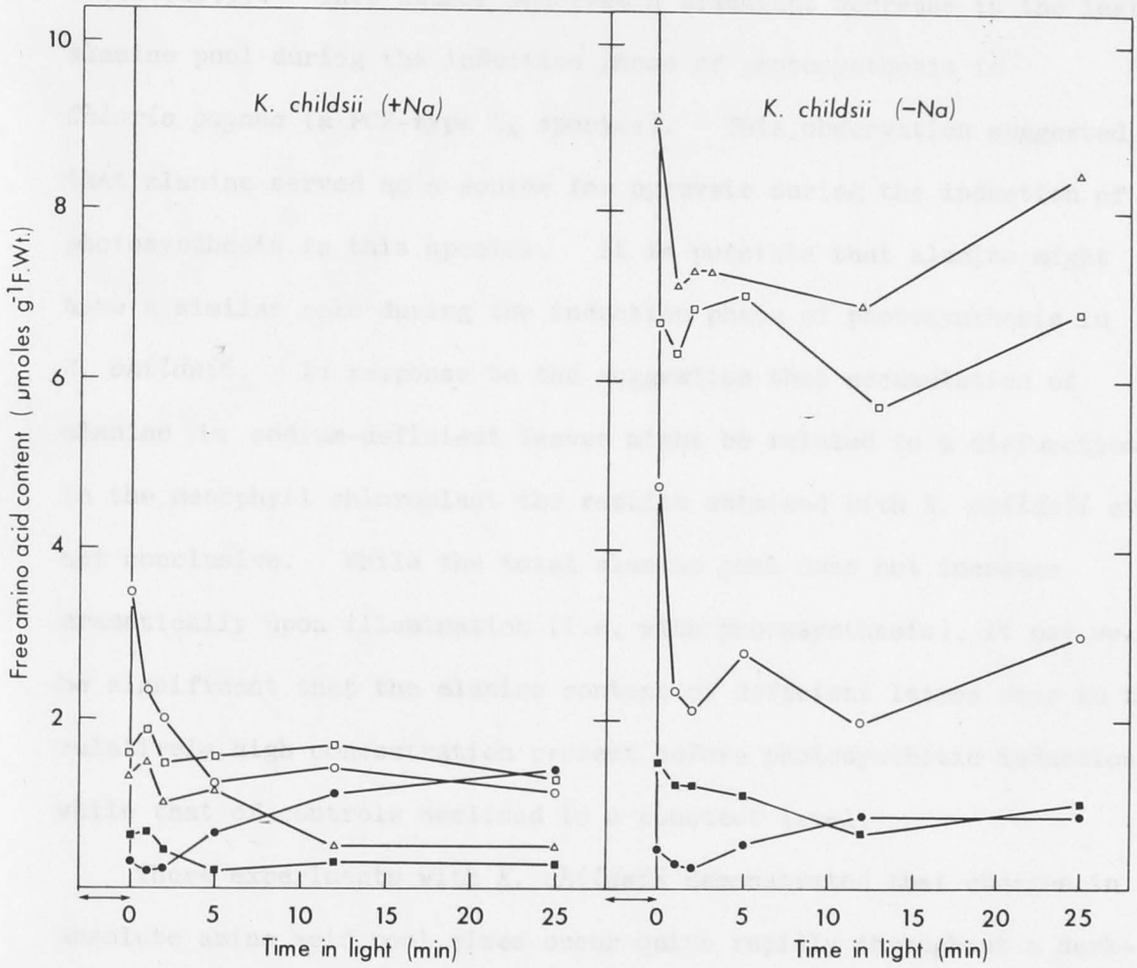


Figure 4.05 The relative changes in the concentrations of alanine (Δ), glycine (●), glutamic acid (□), serine (o) and aspartic acid (■) after illumination in the leaves of sodium-deficient (-Na) and control plants (+Na) of *K. childsii*. Leaves were pre-equilibrated in the dark for a period of 45 minutes (↔) prior to exposure to light (Irradiance was 1 mE m^{-2} s^{-1} (400 - 700 nm)).

concentrations of alanine upon illumination, in sodium-deficient and control leaves, is of interest in relation to results obtained by Hatch (1979). This author observed a transient decrease in the leaf alanine pool during the induction phase of photosynthesis in *Chloris gayana* (a PCK-type C_4 species). This observation suggested that alanine served as a source for pyruvate during the induction of C_4 photosynthesis in this species. It is possible that alanine might have a similar role during the induction phase of photosynthesis in *K. childsii*. In response to the suggestion that accumulation of alanine in sodium-deficient leaves might be related to a disfunction in the mesophyll chloroplast the results obtained with *K. childsii* are not conclusive. While the total alanine pool does not increase dramatically upon illumination (i.e. with photosynthesis), it may well be significant that the alanine content of deficient leaves rose to the relatively high concentration present before photosynthetic induction, while that of controls declined to a constant level.

These experiments with *K. childsii* demonstrated that changes in absolute amino acid pool sizes occur quite rapidly throughout a dark-light transition. This suggested that comparisons between the amino acid contents of leaves which had been sampled during the day, and those sampled shortly after the end of the normal photoperiod *might* serve as an approximate index in differentiating photosynthetic and non-photosynthetic pools. Accordingly, leaf samples were taken, from the same population of plants described in Tables 4.01 and 4.02, approximately 2 - 3 hours after the end of the photoperiod. In Tables 4.03 and 4.04 the amino acid content of these "dark" samples are compared to those obtained from leaves harvested during the day (i.e. designated as "light" samples).

In sodium-deficient and control leaves of all species of absolute

Table 4.03 The concentrations of free amino acids (μmol g^{-1} dry wt) in leaves of sodium-deficient and control plants of *K. childsii*, *A. inflata* and *A. edulis*, sampled in the light and in the dark. Each value is the mean of three replicates and the significance (Students' t-test) between the light (L)[d] and dark (D) treatments is indicated by: a = P 0.001; b = P 0.01; c = P 0.05. (+Na) control; (-Na) sodium-deficient plant.

Amino Acid	*K. childsii*				*A. inflata*				*A. edulis*			
	+Na		-Na		+Na		-Na		+Na		-Na	
	L	D	L	D	L	D	L	D	L	D	L	D
Asp	2.2	7.3[a]	21.2	21.6	9.0	10.9	4.1	14.9[a]	7.6	7.4	4.1	10.4[c]
Ser	21.5	52.5[c]	48.9	67.4[c]	30.8	31.4	20.2	24.3	35.5	24.5	47.7	67.5
Glu	13.3	18.5[c]	97.8	95.0	44.7	39.5	56.6	48.1	48.0	41.2	87.3	60.3
Gly	31.1	4.0[b]	34.5	7.0[a]	6.8	2.3	7.5	1.1[a]	2.3	0.6[c]	31.0	7.2[c]
Ala	4.0	15.5[b]	140.4	128.4	36.1	21.8	61.5	34.4[c]	17.9	25.4[a]	15.9	38.1[a]
TOTAL	88.4	120.4[c]	355.5	331.0	164.8	134.4[b]	220.4	190.4	114.3	111.1	189.7	187.6

[d] Light data are taken from Table 4.01 and were from the same population of plants as the dark samples.

Table 4.04 The concentrations of free amino acids (μmol g^{-1} dry wt) in leaves of sodium-deficient and control plants of *E. crus-galli*, *E. indica* and *C. barbata*, sampled in the light and in the dark. Each value is the mean of three replicates and the significance (Students' t-test) between the light (L)[d] and dark (D) treatments is indicated by: a = P 0.001; b = P 0.01; c = P 0.05. (+Na) control; (-Na) sodium-deficient plant.

Amino Acid	*E. crus-galli*				*E. indica*				*C. barbata*			
	+Na		-Na		+Na		-Na		+Na		-Na	
	L	D	L	D	L	D	L	D	L	D	L	D
Asp	4.5	6.7	8.4	9.3	28.2	29.1	9.4	36.9^{b}	21.5	49.4^{a}	7.1	41.3^{b}
Ser	11.2	13.1	58.5	40.9	64.3	45.1^{c}	57.3	42.0^{c}	111.6	113.8	97.6	117.1^{a}
Glu	6.6	14.6^{a}	39.2	30.0^{b}	61.3	1.5^{a}	74.8	33.3^{b}	65.0	69.1	47.9	50.8
Gly	3.4	2.2	30.6	10.4^{b}	14.6	4.3	44.5	9.8^{b}	29.0	2.6^{a}	34.5	7.7^{a}
Ala	10.0	10.2	220.8	70.7^{a}	48.8	41.7	149.4	76.0^{a}	121.3	51.8^{a}	174.6	59.6^{a}
TOTAL	38.1	51.6	363.9	166.9^{b}	226.5	131.6^{a}	348.8	227.5^{b}	388.9	330.0^{c}	399.3	316.9^{c}

[d] Light data are taken from Table 4.02 and were from the same population of plants as the dark samples.

concentration of the glycine pool was higher in the light than in the dark. This is consistent with the results obtained for *K. childsii* in dark-light transition experiments. However a corresponding decrease in the serine pool in the light is not evident. In the sodium-deficient leaves of several species the concentration of alanine is significantly higher in the light. In control leaves of *C. barbata* the concentration of alanine was also higher in the light suggesting that an increase in alanine concentration in the light is not necessarily a function of sodium nutrition. In sodium-deficient and control leaves of *A. edulis* leaf alanine content is lower in the light. Similarly, Nable (1979) established that the total leaf alanine concentration of sodium-deficient and control plants of *Amaranthus tricolor* increased in the dark. While data presented in Tables 4.03 and 4.04 intimate a general increase in free protein amino acids, and in particular alanine, in leaves of sodium-deficient plants in the light; further speculation would be unwise. The diurnal changes in free amino acid pool sizes are clearly dependent upon an array of metabolic processes.

The magnitude of total free amino pool sizes of sodium-deficient leaves are consistent with the accumulation of label observed in radiotracer kinetic experiments discussed in the previous section (see Section 4.3.1). However, the quantitative significance of amino acid labelling in terms of photosynthetic fluxes can be shown to be negligible. For example, where it is assumed that the maximum photosynthetic rate of a sodium-deficient plant is 25 μmol m^{-2} s^{-1} (i.e. comparable with typical rates obtained in gas exchange experiments in Chapter 3) it would only require approximately 0.3 hours of photosynthesis to assimilate carbon equivalent to a 300 μmol g^{-1} Dry wt free protein amino acid pool (i.e. assuming total pool is comprised of

alanine). This is a simplification but does imply that the nett flow of carbon into free protein amino acids does not represent a substantial drain on photosynthate in sodium-deficient plants.

4.4 SUMMARY

The (^{14}C) labelling kinetics observed in sodium-deficient leaves during simple steady state pulse-chase and time-course experiments are entirely consistent with C_4 photosynthesis. Sodium-deficient plants were characterised by increased accumulation of (^{14}C) label into free protein amino acids. This would appear to be consistent with relatively higher amino acid pools in the leaves of these plants. While the magnitudes of total free protein amino acid pools were identified they are difficult to relate to steady-state photosynthetic metabolism. The accumulation of free amino acids might intimate specific disfunctions in the C_4 metabolism in these plants. However, analyses of total leaf pools did not qualify such speculation.

CHAPTER 5

ENZYMES ASSOCIATED WITH C_4 PHOTOSYNTHESIS IN SODIUM-DEFICIENT PLANTS

5.1 INTRODUCTION

The determination of enzyme activities associated with C_4 photosynthesis and the resolution of their inter- and intracellular location have been important in defining C_4 photosynthetic metabolism (for reviews see Hatch 1976a, b, Hatch and Osmond 1976, Edwards and Huber 1979, Rathnam and Chollet 1980).

A suggested role for sodium in C_4 plants is that it might be required of either the synthesis, or activation of enzyme(s) involved in C_4 photosynthesis (Brownell 1979). As all three C_4 decarboxylating sub-groups show a requirement for sodium (see Section 2.3.2) then sodium could only affect those enzymes which have a common and critical role in all C_4 plants. These are: phosphoenolpyruvate carboxylase, pyruvate, Pi dikinase, adenylate kinase and pyrophosphatase. (Section 1.3.4)

Of these enzymes only phosphoenolpyruvate carboxylase has been studied in the context of sodium deficiency. Holtum (1975) and Brownell (1979) did not detect any differences in the *in vitro* activities of PEP carboxylase extracted from sodium-deficient and control leaves of

K. childsii and *E. utilis*. These authors concluded that sodium nutrition does not affect the formation of PEP carboxylase in C_4 plants. As no measures were taken to obtain sodium-free assay conditions in these experiments the possibility that sodium may be necessary for the activation of this enzyme *in vivo* still exists. However, an activation effect of sodium on PEP carboxylase, pyruvate, Pi dikinase or adenylate kinase could not be detected in assay medium containing less than 0.2 mM sodium (M. D. Hatch, personal communication).

In this chapter the activities of several enzymes involved in C_4 photosynthesis are determined in whole leaf extracts from sodium-deficient and control plants of *E. crus-galli*, *C. barbata* and *E. indica*. In response to the suggestion that sodium might be required for the synthesis or activation of pyruvate, Pi dikinase, the capacities for substrate-dependent O_2 evolution by isolated mesophyll chloroplasts from sodium-deficient and control leaves of *D. sanguinalis* are examined. Isolation experiments were carried out in collaboration with Dr. D. Day.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Sodium-deficient and control plants of *Amaranthus edulis* Speg, *Chloris barbata* Swartz, *Digitaria sanguinalis* (L) Scop, *Echinochloa crus-galli* var. frumentaceae (Roxb) and *Eleusine indica* (L) Gaertn. were obtained using procedures for germination and growth of plants under low sodium conditions described previously (see Sections 2.2.1 - 7). All plants were grown in a naturally illuminated glasshouse under full sunlight (see Section 2.2.2(c)). At the time of the experiments the ages of the plants were approximately those given previously (see

Section 2.3.2, Table 2.2). In all experiments sodium-deficient and control plants were of the same age and only young fully expanded leaves were used.

Biochemicals and reagent enzymes were obtained from Calbiochem (Sydney), Sigma Chemical Co. (St. Louis, Mo) or Boehringer Mannheim Australia (Melbourne) and Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden). Pectinase (Macerozyme R-10) and cellulase (Onozuka cellulysin S-3) were obtained from Kinki Yakult Manufacturing Co. (Japan). Purified PEP-carboxylase, used in assays of Pyruvate, Pi dikinase, was gratefully received from Dr. M. D. Hatch.

5.2.2 Preparation of leaf extracts

Depending upon the enzyme(s) to be assayed one of three methods was used in the preparation of leaf extracts. To minimise experimental error, a procedure was adopted in which both the extraction and assay of individual enzymes of the control and sodium-deficient treatments were carried out sequentially (i.e. controls preceded sodium-deficient treatments and were typically less than three minutes apart).

In the first instance plants were illuminated in the laboratory for at least 45 min (irradiance was 1 mE m^{-2} s^{-1}) prior to enzyme extraction. Approximately 2 g of leaf tissue was rapidly extracted in a chilled mortar and pestle with acid-washed sand and 2 - 3 volumes (w/v) of 50 mM HEPES-KOH buffer, pH 7.5, containing 10 mM Mg Cl_2, 10 mM DTT and 2 mM KH_2PO_4. This homogenate was filtered through Miracloth (Calbiochem), an aliquot taken for chlorophyll determination, and the remaining filtrate centrifuged at room temperature for 2 min at 3000×g. All subsequent steps were carried out at room temperature. Approximately 2.5 ml of the resultant supernatant was then treated on a 4 ml column of Sephadex G-25

previously equilibrated with the extraction buffer. The first 0.2 ml of the chlorophyll-containing band was discarded and the next 1.8 ml collected and then divided into two fractions. The first fraction was kept on ice and the aliquots were taken to determine the activities of PEP carboxylase, NADP-malic enzyme, NAD malate dehydrogenase, NADP malate dehydrogenase and PEP-carboxykinase. The second fraction was warmed to room temperature and an aliquot was taken to determine the initial activity of pyruvate, P_i dikinase. This fraction was then gassed with nitrogen and incubated at room tempearture with 2.5 mM K_2HPO_4 and 6 mM pyruvate for 90-120 minutes to complete the activation of pyruvate p_i dikinase (Hatch and Slack 1969).

For the extraction of aspartate aminotransferase and alanine aminotransferase; 1 - 2 g of leaf tissue was ground in a chilled mortar and pestle with acid-washed sand and 2 volumes (w/v) or 50 mM HEPES-KOH buffer, pH 8.0, containing 10 mM DTT, 20 μg ml^{-1} pyridoxal phosphate and 1 mM EDTA. The homogenate was filtered through Miracloth, an aliquot taken for chlorophyll determination, and samples of the supernatant solution obtained by centrifuging at 12000 x g for 5 min (at $0^{\circ}C$) were used for enzyme assays.

In the extraction of RuP_2 carboxylase; 1 - 2 g of leaf tissue was ground in a chilled mortar and pestle with acid-washed sand and 2 volumes (w/v) of 50 mM HEPES-KOH buffer, pH 7.0, containing 10 mM DTT, 5 mM $MgSO_4$, 1 mM EDTA and 10 mM $NaHCO_3$. The homogenate was filtered through Miracloth, an aliquot taken for chlorophyll determination and samples of the supernatant solution obtained by centrifuging at 3000 x g, for 5 min at room temperature, were used for RuP_2 carboxylase assays.

The chlorophyll content of extracts were determined using the procedure of Arnon (1949).

5.2.3 Assay of enzymes

The following enzymes were measured at 28°C by following the change in absorbance of a pyridine nucleotide at 340 nm in a 1 ml reaction mixture containing 10 or 20 µl of leaf extract. Enzymes were assayed using a Perkin Elmer Double Beam Spectrophotometer (Model 124) according to the following referenced procedures:

PEP carboxylase - (orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31)(Hatch and Oliver 1978)

NADP-malic enzyme - (L-malate:NADP oxidoreductase (decarboxylating), EC 4.1.1.40) (Hatch and Mau 1977)

NAD malate dehydrogenase - (L-malate:NAD oxidoreductase, EC 1.1.1.37) (Johnson and Hatch 1970)

NADP malate dehydrogenase - (L-malate:NADP oxidoreductase, EC 1.1.1.82) (Johnson and Hatch 1970)

Aspartate aminotransferase - (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) (Hatch and Mau 1973)

Alanine aminotransferase - (L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2) (Hatch and Mau 1973)

Pyruvate, Pi dikinase - (ATP:pyruvate, orthophosphate phosphotransferase, EC 2.7.9.1) (Andrews and Hatch 1969).

The enzyme PEP carboxykinase (ATP:oxaloacetate carboxy-lyase (transphosphorylating) EC 4.1.1.49) was assayed in the direction of oxaloacetate decarboxylation by following the decrease in oxaloacetate absorbance at 280 nm according to Hatch (1973). The molar extinction coefficient for oxaloacetate, at the pH and metal ion concentrations used in the assay, was determined on a CAREY 14 recording spectrophotometer.

The enzyme RuP_2 carboxylase (3-phospho-D-glycerate carboxy-lyase

(dimerising) EC 4.1.1.39) was assayed according to the radiochemical method of Lorimer *et al.* (1977) as modified by Wong (1979b). The assay was initiated by adding 20 µl of the leaf extract to a reaction mixture containing 100 mM Tricine-KOH (pH 8.1) 20 mM $MgCl_2$, 1 mM RuP_2 and 20 mM $NaH^{14}CO_3$ (0.5 mCi $mmol^{-1}$) in a total volume of 0.5 ml. After 60 seconds the reaction was stopped by adding 100 µl of 2N HCl, the reaction vial was blown dry using compressed air, and the acid-stable radioactivity was estimated by scintillation counting.

All enzyme activities were calculated on the basis of the chlorophyll content of the crude extract.

Relationships between total leaf chlorophyll and total water-soluble protein were determined on leaves of sodium-deficient and control plants of *C. barbata* and *E. indica*, comparable to those used in enzyme extraction, and on young fully expanded leaves of *A. edulis* and *D. sanguinalis*. From 2 g of leaf material sub-samples were taken for the estimation of total leaf chlorophyll (see Section 2.2.7) and for the determination of total water-soluble protein. In protein extraction samples were ground in a chilled mortar and pestle with acid-washed sand and 5 volumes (w/v) of 50 mM HEPES-KOH buffer (pH 7.5). The resultant homogenate was centrifuged at 10000×g for 10 min at $0^{\circ}C$. The pellet was re-suspended in extraction buffer, re-centrifuged and supernatants were pooled. Aliquots of the resultant supernatant were taken for estimates of total protein using the method of Lowry *et al.* (1951).

5.2.4 Isolation of mesophyll protoplasts from *D. sanguinalis*

The method used for mesophyll protoplast isolation was essentially that of Day *et al.* (1981). Approximately 5 g of leaves from sodium-

deficient or control plants of *D. sanguinalis* were cut transversely with a hand-held razor blade into segments approximately 1 mm wide. Leaf segments were then digested in 10 volumes (w/v) of isolation medium containing 10 mM MES buffer, pH 5.5, 0.5 M sorbitol 1 mM KH_2PO_4, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1% (w/v) BSA, 0.1% (w/v) for sodium-deficient leaves or o.2% (w/v) pectinase (Macerozyme R-10) for controls and 2% (w/v) cellulase (Onozuka S-3), for either 2.5 hours (sodium-deficient leaves) or 3.5 hours (control leaves) at 30°C under low light. Examination of the progressive digestion by light microscopy, during preliminary experiments, established optimal digestion times for sodium-deficient or control leaves. Following digestion, the enzyme medium was decanted and leaf slices were gently agitated in 50 ml of medium A (i.e.50 mM HEPES-KOH (pH 7.5) containing 0.5 M sorbitol, 1 mM $MgCl_2$, 1 mM KH_2PO_4, 0.5% (w/v) BSA). This suspension was then filtered through a coarse mesh nylon strainer and the resulting mixture of bundle-sheath strands, cells, cell debris and protoplasts was successively filtered through nylon meshes of 500, 350 and 80 µm. The resultant filtrate was centrifuged at 300 g for 3 min and the pellet gently re-suspended in medium B (i.e. this was identical to medium A except that sucrose replaced sorbitol). Four 50 ml glass centrifuge tubes were used and pellets were re-suspended in 5 ml of medium B, overlaid with 2 ml of medium A, and centrifuged for 5 min at 350 g. The mesophyll protoplasts aggregated at the sucrose-sorbitol interface and were carefully removed with a Pasteur pipette. The mesophyll protoplasts thus isolated were concentrated by re-centrifuging at 350 g for a further 3 min and the resultant pellet was re-suspended in 50 mM Tricine buffer (pH 8.0) containing 0.33 M sorbitol and 10 mM EDTA and then stored on ice. Protoplasts were disrupted by sucking through a 20 µm nylon mesh fitted to a 1 ml

syringe (three passes). This was designated as the protoplast extract and was used in measurements of substrate-dependent O_2 evolution. All isolation steps were performed at room temperature.

5.2.5 Oxygen evolution by protoplast extracts

Substrate-dependent oxygen evolution was measured polarographically at 30°C with a Clark O_2-electrode system. Approximately 20 µg of chlorophyll was added to 2 ml of reaction medium containing 0.3 M Sorbitol, 50 mM Tricine (pH 8.0), 10 mM EDTA and 0.5 mM KH_2PO_4. Catalase (20 IU) was routinely added to the reaction medium. Illumination was provided by a Hanimex 2300S projector and could be altered by interposing calibrated neutral density filters (Balzers, Leichenstein). Irradiance was measured with a quantum sensor (Lambda Instruments, model IL-190SR) at the surface of the electrode vessel. In routine experiments irradiance was approximately 1400 µE m^{-2} s^{-1} (400 - 700 nm). Details of the substrates used, and their concentrations, are given in the figure legends.

5.3 RESULTS AND DISCUSSION

5.3.1 Activities of enzymes associated with C_4 photosynthesis in sodium-deficient plants

The activities of some enzymes, associated with C_4 photosynthesis, in whole leaf extracts from sodium-deficient and control plants of *E. crus-galli* (an NADP-ME-type), *C. barbata* (a PCK-type) and *E. indica* (an NAD-ME-type) are given in Table 5.01. All enzyme activities were calculated on the basis of the chlorophyll content of crude extracts (Section 5.5.2). The maximum rates of photosynthesis exhibited by

Table 5.01 The activities of enzymes associated with C_4 photosynthesis in leaves of sodium-deficient and control plants of *E. crus-galli* (NADP-ME type), *C. barbata* (PCK-type) and *E. indica* (NAD-ME type). [a]

	Activity in whole leaf extracts (μmoles min^{-1} (mg chlorophyll)$^{-1}$)					
	E. crus-galli		*C. barbata*		*E. indica*	
	+Na	-Na	+Na	-Na	+Na	-Na
RuP_2 carboxylase	6.8	8.5	7.3	6.7	6.8	8.3
	9.3	8.2	9.9	6.9	7.5	8.9
PEP carboxylase	30.1	57.3	88.4	128.0	30.2	58.5
	34.3	63.9	50.1	100.0	35.5	66.7
NADP malic enzyme	12.6	6.8	0.5	1.7	0.6	2.6
	11.5	9.4	0.5	1.0	0.9	1.8
PEP carboxykinase	0.2	0.2	7.3	5.5	0.1	N.D.
	-	-	-	-	-	-
Pyruvate, Pi dikinase [bd]	5.7	10.2	5.4	10.0	4.5	15.2
	3.3	10.5	3.6	11.3	7.5	15.4
Alanine aminotransferase	0.9	7.1	12.1	38.2	166.3	264.0
	1.5	5.6	17.5	35.1	151.5	232.6
Aspartate aminotransferase	6.0	8.1	25.2	90.1	84.9	112.1
	4.2	8.4	32.3	97.5	75.9	120.3
NAD malate dehydrogenase	82.8	109.9	216.4	344.1	61.6	165.2
	77.8	112.9	143.1	265.8	71.5	143.6
NADP malate dehydrogenase	4.0	4.5	0.7	0.9	0.8	0.8
	2.8	5.2	0.6	0.9	1.1	1.5
$^*A_{max}$ ($^*A$,I) [c]	8.7	7.0	8.1	5.4	8.9	5.4

[a] For each enzyme the results of two independent experiments are shown.

[b] Activated *in vitro* for 90-120 min prior to assay.

[c] $^*A_{max}$ is the light-saturated rate of CO_2 assimilation (i.e. irradiance 1700 μE m^{-2} s^{-1}, 21% O_2, P_a = 330 μbar CO_2) of sodium-deficient and control leaves as expressed on a total leaf chlorophyll basis (see Chapter 3, Section 3.3.2, Figure 3.02). Gas exchange measurements were made on leaves comparable to those used for enzyme extraction.

[d] In both sodium-deficient and control extracts, initial activities of pyruvate, Pi dikinase were typically 70-80% of the *in vitro* activated rates.

comparable leaves, expressed on the basis of total leaf chlorophyll content (Chapter 3, Section 3.3.2), are included in Table 5.01 for comparison.

In all of the species examined the activities of PEP carboxylase, the enzyme associated with the primary carboxylation of atmospheric CO_2 and located in the cytoplasm of mesophyll cells in all C_4 species (Hatch *et al.* 1975), are relatively higher in the leaves of sodium-deficient plants. The activities of this enzyme obtained in control leaves are comparable to those recorded for other C_4 species (Hatch and Osmond 1976). For both sodium-deficient and control leaves *in vitro* activities of PEP carboxylase are in excess of the maximum rates of photosynthesis. This would suggest that PEP carboxylase does not limit the photosynthetic capacity of sodium-deficient plants (see also Chapter 3, Section 3.3.5). Holtum (1975) and Brownell (1979) concluded that the synthesis of PEP carboxylase is not directly affected by sodium nutrition and the results obtained in this study confirm this conclusion.

The activities of RuP_2 carboxylase, which is localised in the chloroplasts of bundle sheath cells in C_4 plants (Hatch *et al.* 1975, Hatch and Osmond 1976) were similar in sodium-deficient and control leaves of *E. crus-galli*, *C. barbata* and *E. indica*. The *in vitro* activities of this enzyme in whole leaf extracts of control plants are comparable to those obtained by Wong (1979a, b) and are somewhat higher than those generally obtained in C_4 plants (Ku *et al.* 1979, Rathnam and Chollet 1980, Hatch and Osmond 1976). The *in vitro* activities of RuP_2 carboxylase recorded for control leaves are comparable to the maximum rates of photosynthesis in these plants. However, *in vitro* activities of RuP_2 carboxylase recorded for sodium-deficient leaves are higher than the maximum photosynthetic rates of these plants. As the C_4 pathway serves to concentrate CO_2 in the vicinity of RuP_2 carboxylase, thereby

optimising the activity of this enzyme (Hatch and Osmond 1976), it is unlikely that the photosynthetic capacity of sodium-deficient plants is limited by the levels of RuP_2 carboxylase (see also Chapter 3, Section 3.3.5). Where *in vitro* activities of RuP_2 carboxylase are compared with *in vitro* activities of PEP carboxylase it is evident that sodium-deficient leaves possess relatively higher activities of PEP carboxylase as compared to controls. The possible implications of this disproportionality were discussed in relation to photosynthetic CO_2 assimilation in a previous section (Chapter 3, Section 3.3.5).

Of the three C_4 decarboxylating enzymes, only NADP malic enzyme and PEP carboxykinase were examined in these experiments. High activities of NADP malic enzyme, localised in the chloroplasts of bundle-sheath cells (Hatch and Osmond 1976) were recorded in leaf extracts from sodium-deficient and control plants of *E. crus-galli*. This is consistent with the previous classification of this species as an NADP-ME-type C_4 plant (Gutierez *et al.* 1974). The *in vitro* activities of this enzyme in control extracts are comparable to those recorded for other NADP-ME-type C_4 species (Hatch *et al.* 1975). In both sodium-deficient and control leaves *in vitro* activities of NADP malic enzyme are higher than the measured photosynthetic rates for these plants. Substantial activities of PEP carboxykinase, which is localised in the cytoplasm of PEP-CK-type C_4 species (Hatch and Osmond 1976, Ku *et al.* 1980), were recorded in leaf extracts of sodium-deficient and control plants of *C. barbata*. This confirms the classification of this species as a PCK-type C_4 plant and is consistent with (^{14}C) labelling (Chapter 4, Section 4.3.1), and ultrastructural characteristics (Chapter 6, Section 6.3.1) of this species. The *in vitro* activities of this enzyme are comparable to the maximum photosynthetic rates of sodium-deficient and control plants.

High activities of pyruvate, Pi dikinase, an enzyme located in the

mesophyll chloroplast of C_4 plants (Hatch *et al.* 1975, Hatch and Osmond 1976), were recorded in leaf extracts from sodium-deficient and control plants of all three species. The activities recorded for control leaves are similar to those generally obtained in C_4 plants (Hatch *et al.* 1975), but are substantially lower than the measured rates of photosynthesis of control plants. Importantly, *in vitro* activities of pyruvate, Pi dikinase recorded in sodium-deficient extracts are higher than the measured photosynthetic rates of these plants. Such high activities of pyruvate, Pi dikinase in sodium-deficient leaves preclude the suggestion that sodium might be directly involved in the synthesis of this enzyme (Brownell 1979). The enzyme pyruvate, Pi dikinase has complex and varying requirements for activation in leaf extracts (Hatch and Slack 1969, Hatch *et al.* 1975) and comparisons of *in vitro* activity and photosynthetic rates in control leaves suggest that extraction and assay procedures were not optimal. As the same extraction and assay procedures were used in determining the activity of this enzyme from sodium-deficient leaves, it is possible that these experiments also underestimate the *in vitro* activity of pyruvate, Pi dikinase from sodium-deficient leaves.

Highest activities of alanine and aspartate aminotransferase were recorded in extracts from sodium-deficient and control leaves of *C. barbata* and *E. indica*. This is consistent with the classification of these species as PCK- and NAD-malic enzyme-types respectively (Hatch *et al.* 1975). In *C. barbata* alanine and aspartate aminotransferase are located in the cytoplasm of both bundle-sheath and mesophyll cells (Hatch *et al.* 1975). In *E. indica* alanine aminotransferase is equally distributed between the cytoplasm of bundle-sheath and mesophyll cells, while aspartate aminotransferase is located in the cytoplasm of mesophyll cells and within bundle-sheath mitochondria (Hatch *et al.* 1975,

Hatch and Osmond 1976). Relatively higher activities of amino-transferases in sodium-deficient leaves are analogous to the higher activities of PEP-carboxylase recorded for these plants and would suggest that sodium deficiency does not result in a substantial reduction in the synthesis of non-chloroplastic enzymes. This suggestion obtains support through comparisons of the *in vitro* activities of NAD-malate dehydrogenase, recorded for sodium-deficient and control leaves. In sodium-deficient leaves the *in vitro* activities of NAD-malate dehydrogenase are relatively higher in each of the species examined. In *E. indica*, the NAD-ME-type C_4 plant, where the bundle-sheath mitochondrial NAD-MDH is implicated in C_4 photosynthesis (Hatch *et al.* 1975), relative differences in activities of the sodium-deficient and control plants were most pronounced. Similar activities of NADP-malate dehydrogenase were recorded for sodium-deficient and control leaves of *E. crus-galli*. This is consistent with the role of this enzyme in C_4 photosynthesis in the mesophyll chloroplasts of NADP-ME-type C_4 plants (Hatch *et al.* 1975). In the control extracts the activities of this enzyme were less than the photosynthetic rates of comparable leaves suggesting that this enzyme may have been inactivated in the course of extraction.

From Table 5.01 it is evident that C_4 photosynthesis in sodium-deficient plants is not limited by the activities of PEP carboxylase, RuP_2 carboxylase and pyruvate, Pi dikinase; three enzymes having a common and critical role in all C_4 plants. Given the inter- and intracellular partitioning of enzymes involved in C_4 photosynthesis, more explicit interpretation of enzyme activities in relation to C_4 photosynthesis in sodium-deficient plants with reference to whole leaf extracts is clearly limited. Higher activities of PEP carboxylase and pyruvate, Pi dikinase recorded in sodium-deficient leaves might suggest

an alteration in the ratio of bundle-sheath:mesophyll compartments in sodium-deficient leaves, i.e. there might be an increase in the relative proportion of mesophyll cells. However, there were no differences in the total leaf chlorophyll $^{a}/b$ ratios of sodium-deficient and control leaves of *D. sanguinalis* and *E. crus-galli* (Chapter 2, Table 2.03). As bundle sheath chloroplasts have fewer grana in these species, and express higher chlorophyll $^{a}/b$ ratios (Mayne *et al* 1971, 1974 Chang and Troughton 1972), a substantial alteration in the ratio of bundle sheath to mesophyll cells should result in a shift in the chlorophyll $^{a}/b$ ratio of the total leaf chlorophyll. Such considerations highlight the need for further research towards identification of the inter- and intracellular activities of C_4 enzymes in sodium-deficient leaves.

The relatively higher activities of non-chloroplastic enzymes recorded in sodium-deficient leaves (Table 5.01) are consistent with the higher soluble protein/chlorophyll ratios recorded for sodium-deficient leaves as shown in Table 5.02. While there is a reduction in the level of soluble protein in sodium-deficient leaves there is a more substantial reduction in total leaf chlorophyll. In the leaves of C_4 plants, levels of PEP carboxylase represent 10 - 15% (Björkman *et al.* 1976, Hague and Sims 1980), and levels of RuP_2 carboxylase represent 10 - 20% of the total soluble protein (Ku *et al.* 1979). Given the reduced level of RuP_2 carboxylase in sodium-deficient leaves (Chapter 3, Table 3.03), it seems possible that this might contribute towards the overall reduction in total soluble protein observed in these leaves. Again, further research is needed to identify the location and composition of the total soluble protein fraction of sodium-deficient and control leaves.

Table 5.02 The relationship between total water soluble protein and total chlorophyll in leaves of sodium-deficient and control plants of *E. indica*, *C. barbata*, *D. sanguinalis* and *A. edulis*.

Species and treatment		Soluble protein[a] ($g\ m^{-2}$)	Total chlorophyll[a] ($g\ m^{-2}$)	$\frac{\text{Total protein}}{\text{Total chlorophyll}}$[b]
E. indica	+Na	3.50(0.17)	0.53(0.02)	6.6
	-Na	2.99(0.10)	0.24(0.01)	12.5
C. barbata	+Na	5.40(0.50)	0.44(0.03)	12.3
	-Na	4.22(0.41)	0.28(0.02)	15.1
D. sanguinalis	+Na	3.59(0.20)	0.54(0.03)	6.7
	-Na	2.82(0.21)	0.31(0.03)	9.1
A. edulis	+Na	4.65(0.54)	0.39(0.03)	11.9
	-Na	4.15(0.39)	0.24(0.01)	17.3

[a] Each value is the mean of three independent determinations. Standard deviations given in brackets.

[b] Ratio of the means.

5.3.2 Oxygen evolution by isolated mesophyll chloroplasts

Mesophyll chloroplasts isolated from sodium-deficient and control leaves of *D. sanguinalis* displayed rates of oxygen evolution within the range 2.3 - 3.8 $\mu mol\ min^{-1}\ (mg\ chl)^{-1}$, with either 3-phosphoglyerate or with oxaloacetate plus pyruvate, as shown in Table 5.03. These rates of O_2 evolution are comparable to those obtained in isolated C_4 mesophyll chloroplasts by other workers (Huber and Edwards 1975 a,b,c, Day *et al.* 1981, Edwards *et al.* 1979).

Table 5.03 Substrate dependent O_2 evolution by mesophyll protoplast extracts from sodium-deficient and control leaves of *D. sanguinalis*. O_2 evolution was measured as described in Section 5.2.5 with 2.5 mM PGA, 0.5 mM OAA and 5 mM pyruvate.

Substrate	Expt.	O_2 evolution (μmol min^{-1} (mg chl)$^{-1}$)	
		+Na	-Na
PGA	1	2.4	3.8
	2	-	3.2
OAA + pyruvate	1	2.3	3.5
	2	2.5	3.1
Ratio; pyruvate/OAA	1	2.5	2.7
	2	2.2	2.7

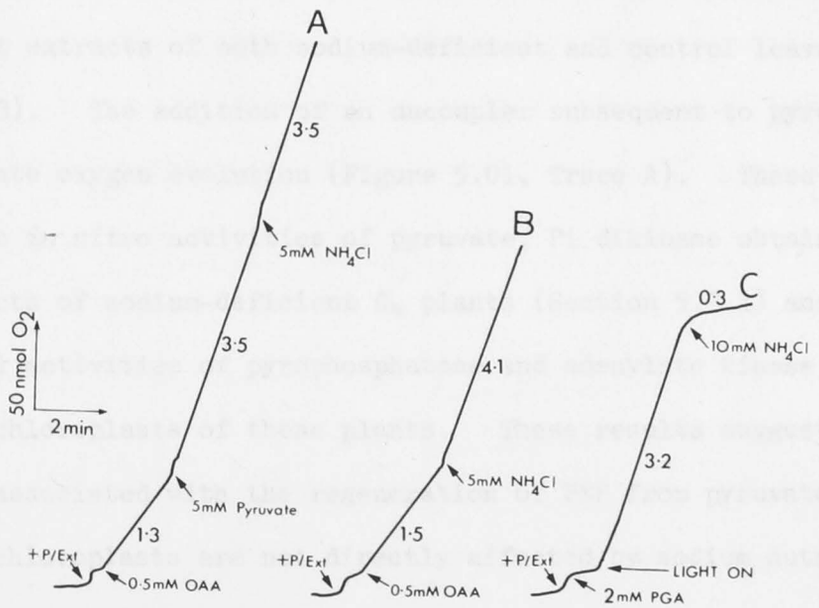


Figure 5.01 Oxygen evolution by mesophyll protoplast extracts from sodium-deficient leaves of *D. sanguinalis*. Protoplast extracts, obtained by disrupting protoplasts (see Section 5.2.4) were added to a concentration of approximately 20 µg chlorophyll. Numbers on traces indicate rates of O_2 evolution in µmol min^{-1} (mg Chl)$^{-1}$.

Representative traces of substrate-dependent O_2 evolution in mesophyll protoplast extracts from sodium-deficient leaves are shown in Figure 5.01. *D. sanguinalis*, an NADP-malic enzyme-type C_4 species, has substantial activity of NADP-malate dehydrogenase in mesophyll chloroplasts (Hatch *et al.* 1975, Hatch 1976b, Hatch and Osmond 1976). In this species OAA-dependent O_2 evolution (Figure 5.01, Trace A, B) is therefore attributable to OAA reduction *via* NADP-malate dehydrogenase. In this instance non-cyclic electron flow is restricted by ATP turnover, hence oxygen evolution is stimulated when either pyruvate (Figure 5.01, Trace A) or an uncoupler, e.g. NH_4Cl (Figure 5.01, Trace B) is added. Typically, addition of pyruvate subsequent to oxaloacetate resulted in a 2 - 3 fold stimulation of oxygen evolution in chloroplast extracts of both sodium-deficient and control leaves (Table 5.03). The addition of an uncoupler subsequent to pyruvate does not stimulate oxygen evolution (Figure 5.01, Trace A). These results confirm the *in vitro* activities of pyruvate, Pi dikinase obtained in leaf extracts of sodium-deficient C_4 plants (Section 5.3.1) and imply substantial activities of pyrophosphatase and adenylate kinase in mesophyll chloroplasts of these plants. These results suggest that the processes associated with the regeneration of PEP from pyruvate in the mesophyll chloroplasts are not directly affected by sodium nutrition *in vivo*.

Rates of oxygen evolution exhibited by chloroplast extracts from sodium-deficient leaves are generally higher than those obtained from control leaves (Table 5.03). As it is generally accepted that prolonged enzyme digestion periods result in lower specific activities of the isolated mesophyll chloroplasts (Huber and Edwards 1975a, Day *et al.* 1981), this difference is probably attributable to the longer digestion periods required for the control leaves (Section 5.2.4).

Mesophyll chloroplasts of C_4 plants are capable of PGA reduction *via* PGA-kinase and GAP dehydrogenase (Hatch 1976a, b, Hatch and Osmond 1976). Consequently, mesophyll chloroplasts from both sodium-deficient and control leaves of *D. sanguinalis* exhibit high rates of PGA-dependent oxygen evolution (Table 5.03, Figure 5.01, Trace C). Rates of PGA-dependent oxygen evolution were inhibited by the addition of high concentrations of NH_4Cl (Figure 5.01, Trace C). Presumably, the addition of high concentrations of uncoupler effectively diminishes ATP production *via* non-cyclic electron flow thereby suppressing the reduction of PGA.

Similar responses of 3-PGA dependent O_2 evolution to irradiance were exhibited by isolated mesophyll chloroplasts from sodium-deficient and control leaves as shown in Figure 5.02. These results further suggest that isolated mesophyll chloroplasts from sodium-deficient and control leaves of *D. sanguinalis*. are functionally similar.

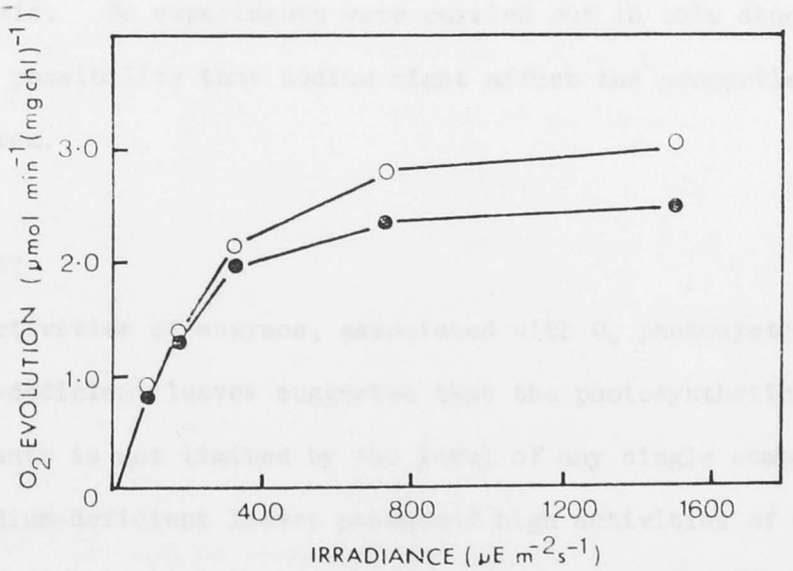


Figure 5.02 The relationship between 3-PGA-dependent oxygen evolution and irradiance in isolated mesophyll chloroplasts from sodium-deficient (o) and control leaves (•) of *D. sanguinalis*.

In these experiments PGA-dependent oxygen evolution was assayed in the presence of relatively low concentrations of inorganic phosphate (Section 5.2.5). It has been reported that high concentrations of inorganic phosphate inhibit PGA-dependent oxygen evolution by C_4 mesophyll chloroplasts, suggesting that 3-PGA and Pi compete for the same carrier (Edwards *et al.* 1979, Day *et al.* 1981). Recent studies have shown that the transport of 3-phosphoglycerate, phosphoenol-pyruvate, inorganic phosphate and dihydroxyacetone phosphate is catalysed by a common carrier - termed the phosphate translocator (Day and Hatch 1981a, b (in press)). Consequently, malate and phosphoenolpyruvate production from OAA and pyruvate, respectively, are inhibited by 3-PGA, the extent of this inhibition being dependent upon the relative concentrations of inorganic phosphate and 3-PGA. These authors suggest that 3-PGA from bundle-sheath cells may serve as a feedback regulator of the mesophyll cell reactions during C_4 photosynthesis. No experiments were carried out in this study to examine the possibility that sodium might affect the properties of this carrier system.

5.4 SUMMARY

The activities of enzymes, associated with C_4 photosynthesis, from sodium-deficient leaves suggested that the photosynthetic capacity of these plants is not limited by the level of any single component enzyme. Sodium-deficient leaves possessed high activities of pyruvate, P_i dikinase which implied that sodium is not required for the synthesis of this enzyme. Furthermore, the capacities for substrate-dependent O_2 evolution, by isolated mesophyll chloroplasts from sodium-deficient leaves, suggested that the conversion of pyruvate to PEP is unimpaired in these organelles.

CHAPTER 6

ULTRASTRUCTURAL FEATURES AND CHLOROPHYLL *a* FLUORESCENCE OF SODIUM-DEFICIENT LEAVES

6.1 INTRODUCTION

Experiments which have been described in the preceding chapters were largely carried out on whole leaves of sodium-deficient and control C_4 plants. However, as C_4 photosynthesis involves "an intricate complex of co-operative processes, based on compartmentation and transport" (Hatch and Osmond 1976), interpretation of whole leaf studies is limited without more explicit information concerning component reactions of the bundle sheath and mesophyll cells. The classification of C_4 species into particular sub-groups on the basis of both biochemical *and* cytological characteristics (Gutierrez *et al.* 1974, Hatch *et al.* 1975), would suggest that examination of the ultrastructural features of the mesophyll and bundle sheath cells of sodium-deficient leaves might enhance our understanding of the photosynthetic basis of sodium deficiency. Accordingly a preliminary examination of the ultrastructural characteristics of sodium-deficient leaves is presented in the first section of this chapter.

An integral feature of plants possessing the C_4 pathway is the compartmentation of component reactions within two distinct chlorenchymatous cell types (Downton 1971, Hatch *et al.* 1975, Black 1973). As C_4 photosynthesis was first correlated with a "Kranz-type" leaf anatomy, i.e. where the photosynthetic tissues are organised in a double hollow cylinder of cells which is wreath-like or "Kranz" in

cross section, these two cell types have been designated mesophyll and bundle sheath. The C_4 pathway involves the rapid bidirectional transport of intermediates between mesophyll and bundle sheath cells, and numerous plasmodesmata, connecting these cell types, are presumed to mediate a diffusive flux of metabolites (Osmond 1971, Osmond and Smith 1976, Hatch and Osmond 1976).

Classification of C_4 plants into several sub-groups (Gutierrez *et al.* 1974, Hatch *et al.* 1975) suggests implicit relationships between the biochemistry of C_4 photosynthesis and the number, distribution and ultrastructure of leaf organelles. For example, bundle sheath chloroplasts of NADP-ME-type C_4 plants have reduced grana. This characteristic has been correlated with a partial photosystem II deficiency which is compensated for by a decarboxylation system donating both CO_2 and NADPH to the bundle sheath cells (Hatch and Osmond 1976, Edwards and Huber 1979). A second example relates to the numerous and large mitochondria observed in the bundle sheath cells of NAD-ME-type C_4 plants. These reflect the direct involvement of the mitochondria in this particular C_4 decarboxylase system (Hatch *et al.* 1975).

The effects of both macro- and micronutrient deficiencies on chloroplast ultrastructure have been examined in a variety of plant species (Vesk *et al.* 1966, Thomson and Weier 1962, Hall *et al.* 1972). In some cases, the fine structure of the chloroplasts in mineral-deficient leaves have been correlated with the photochemical capacities of isolated chloroplasts *in vitro* (Baszynski *et al.* 1972, Terry 1980). However, interpretation of causal connections between changes in the ultrastructure of chloroplasts, and the role of a particular mineral nutrient remain speculative in many instances.

In the second part of this chapter chlorophyll *a* fluorescence

transients from intact, attached leaves of sodium-deficient and control plants are presented. In photosynthetic research, chlorophyll *a* fluorescence induction has been used to obtain information on excitation energy transfer, the size of electron acceptor pools of photosystem II and the electron flow on either the electron donor or acceptor side of photosystem II (for review see Papageorgiou 1975). Differences in chlorophyll *a* fluorescence transients of intact leaves reflect intrinsic differences in the photosynthetic machinery of sun and shade plants (Fork and Govindjee 1980) and improved fluorescence induction methods have recently provided quantitative estimates of photosystem II photosynthetic unit sizes in a variety of higher plants (Malkin *et al.* 1981, Malkin and Fork 1981). Comparisons of the chlorophyll *a* fluorescence of sodium-deficient and control leaves could reveal any severe disfunction in the primary photochemical processes, as a result of sodium deficiency. The measurement of chlorophyll *a* fluorescent transients were made in collaboration with Dr. Christa Critchley.

6.2 MATERIALS AND METHODS

6.2.1 Plant material

Sodium-deficient and control plants of *E. crus-galli*, *E. indica* and *C. barbata* were grown in a naturally illuminated glasshouse (Section 2.2.2C) using the procedures for growth of plants under low-sodium culture conditions outlined in a previous chapter (Chapter 2, Section 2.2.3 to 2.2.5). Tissue samples for ultrastructural examination, and fluorescence measurements, were taken from leaves comparable to those used in gas exchange experiments (Chapter 3, Section 3.2.1).

6.2.2 Transmission electron microscopy

Sodium-deficient and control plants of *C. barbata* and *E. indica* were kept in the dark for approximately 14 hours (overnight) prior to fixation. All steps were carried out at room temperature unless otherwise specified.

Tissue blocks, approximately 1 mm^2, were taken from the mid-region of young fully expanded leaves and were fixed for 1 hour in the primary fixative - 3% paraformaldehyde, 3% glutaraldehyde in 50 mM phosphate buffer (pH 6.8). The fixative was changed and tissue blocks were left at 5°C for a further 12 hours. Tissue blocks were then rinsed in three 20 min. changes of 50 mM phosphate buffer (pH 6.8) and post-fixed for 2 hours in 2% osmium tetroxide in 50 mM phosphate buffer (pH 6.8). This secondary fixative was then removed and tissue blocks were rinsed with distilled water. Specimens were then dehydrated in an acetone series and embedded in Spurr's low viscosity embedding medium.

Thick, i.e. 1 - 2 µm, and ultrathin sections were cut using glass knives on a Reichert UM2 ultramicrotome. Thick sections were stained with toluidine blue and examined under the light microscope. Ultrathin sections were stained with saturated aqueous uranyl acetate (30 min.) and Reynold's lead citrate (20 min.) and then examined in a Hitachi 500 Transmission Electron Microscope.

6.2.3 Fluorescence measurements

Chlorophyll *a* fluorescence induction kinetics of intact, attached leaves of sodium-deficient and control plants of *E. crus-galli*, *E. indica* and *C. barbata*, were measured with a Plant Productivity Fluorometer, Model SF10 (Richard Crancker Research Ltd., Canada). Quantum flux density was 30 µE m^{-2} s^{-1}, centering around 670 nm, which was sufficient

to induce electron transport. The fluorescence transients were recorded on a Tektronix 564 storage oscilloscope and photographed. Experiments were carried out at room temperature (ca. 25°C) and all measurements were taken from the upper surface of leaves after dark adaptation overnight.

6.3 RESULTS AND DISCUSSION

6.3.1 Anatomical and ultrastructural observations

Some anatomical and ultrastructural features of sodium-deficient and control leaves of *C. barbata* and *E. indica* are shown in Figures 6.01 and 6.02 respectively. The leaves of sodium-deficient plants used in these experiments were visibly chlorotic, i.e. the total leaf chlorophyll contents were one-half to one-third of the control leaves and leaf chlorophyll $^{a}/_{b}$ ratios of sodium-deficient and control plants were similar in both species (Section 2.3.2, Table 2.03).

"Kranz-type" leaf anatomy is evident in both sodium-deficient and control leaves of *C. barbata* and *E. indica* (Figure 6.01a, d and 6.02a, d). In *C. barbata* bundle sheath chloroplasts are located centrifugally in both sodium-deficient and control leaves. This feature is characteristic of PCK-type C_4 grasses (Gutierrez *et al.* 1974, Hatch *et al.* 1975). In sodium-deficient and control leaves of *E. indica*, bundle sheath chloroplasts are located centripetally as is characteristic of NAD-ME-type C_4 grasses (Gutierrez *et al.* 1974, Hatch *et al.* 1975). The sodium-deficient leaves of both species appear thinner in cross-section, confirming visual observations and qualifying the higher specific leaf areas shown for these plants (Section 2.3.2, Table 2.03). However the extent of such differences in leaf thickness

Figure 6.01 Anatomical and ultrastructural features of control (Plates a, b and c) and sodium-deficient (Plates d, e and f) leaves of *Chloris barbata*. All scale bars are given in µm.

Plate a Light micrograph of control leaf (transverse section) showing bundle-sheath cells (BS) with chloroplasts tending towards the centrifugal position, mesophyll cells (M) and vascular tissue (V).

Plate b Electron micrograph of control mesophyll of chloroplasts (chl) with prominent grana (g) and peripheral reticulum (pr).

Plate c Electron micrograph of control bundle-sheath cell, showing the distribution of chloroplasts (chl) and mitochondria (mit)

Plate d Light micrograph of sodium-deficient leaf (transverse section) showing bundle-sheath cells (BS) with chloroplasts tending towards the centrifugal position, mesophyll cells (M) and vascular tissue (V). Compared to the control (a) the leaf is thinner, and cells are generally smaller.

Plate e Electron micrograph of sodium-deficient mesophyll chloroplasts (chl). In comparison with the control (b) there are fewer grana (g) per chloroplast and prominent regions of unappressed lamellae (u/a). Peripheral reticulum (pr).

Plate f Electron micrograph of sodium-deficient bundle-sheath cell showing distribution of chloroplasts (chl) and mitochondria (mit). In comparison with the control (c) there appear to be fewer thylakoid membranes per chloroplast and regions of unappressed lamellae are again evident

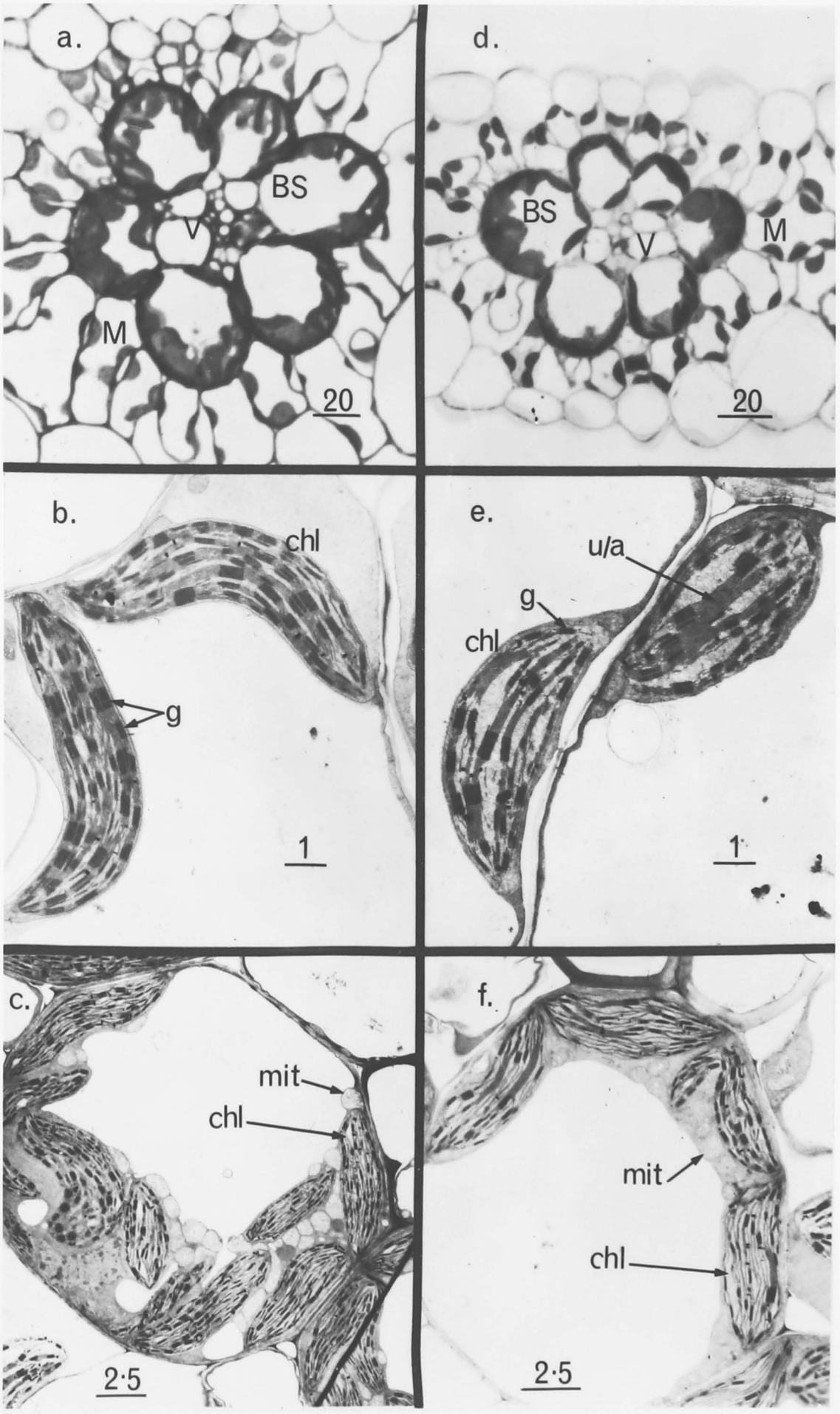

a.
BS
V
M
20
d.
BS
V
M
20
b.
chl
g
1
e.
u/a
g
chl
1
c.
mit
chl
2·5
f.
mit
chl
2·5

Figure 6.02 Anatomical and ultrastructural features of control (Plates a, b and c) and sodium-deficient (Plates d, e and f) leaves of *Eleusine indica*. All scale bars are given in µm.

Plate a Light micrograph of control leaf (transverse section) showing bundle-sheath cells (BS) with centripetally located chloroplasts, mesophyll cells (M) and vascular tissue (V).

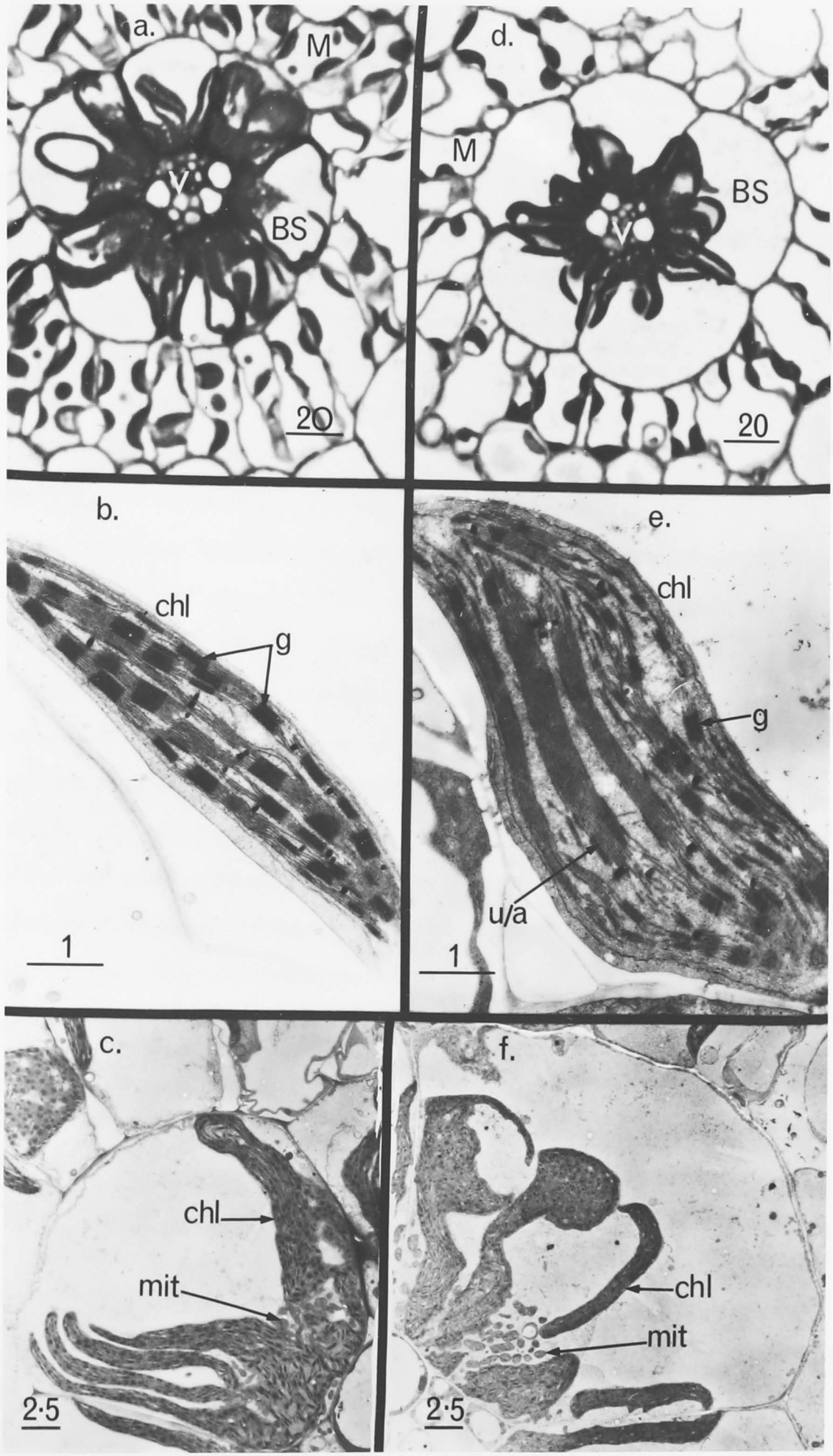
Plate b Electron micrograph of control mesophyll chloroplast (chl) with prominent grana (g)

Plate c Electron micrograph of bundle-sheath cell showing the distribution of chloroplasts (chl) and mitochondria (mit)

Plate d Light micrograph of sodium-deficient leaf (transverse section) showing bundle-sheath cells (BS) with centripetally located chloroplasts, mesophyll cells (M) and vascular tissue (V). Compared to the control (a), the leaf is thinner.

Plate e Electron micrograph of sodium-deficient mesophyll chloroplast (chl) with grana (g) and prominent regions of unappressed lamellae (u/a)

Plate f Electron micrograph of sodium-deficient bundle-sheath cell showing the distribution of chloroplasts (chl) and mitochondria (mit)


a.
M
V
BS
20
d.
M
BS
V
20
b.
chl
g
1
e.
chl
g
u/a
1
c.
chl
mit
2·5
f.
chl
mit
2·5

were dependent upon vascular bundles examined, i.e. the distance of the vascular bundles from the mid-rib. At the ultrastructural level, there was variation in the chloroplast population of the leaf samples from both sodium-deficient and control leaves. Those leaf organelles shown in Figures 6.01 and 6.02, and described in the following discussion, were representative of the chloroplast populations examined.

Ultrastructural features of mesophyll chloroplasts and bundle sheath cells from sodium-deficient and control leaves of *C. barbata* are shown in Figures 6.01b, c and 6.01 e, f respectively. Mesophyll chloroplasts of control and sodium-deficient leaves have a well developed system of granal and stromal lamellae (Figure 6.01 b, e). Mesophyll chloroplasts of sodium-deficient leaves are only marginally smaller than those of controls, i.e. assessed by the length of the longest axis, but appear swollen or distended (see also *E. indica* Figure 6.02 b, e). While the same fixation and embedding schedule was used for both deficient and control samples, the possibility that this swollen appearance is an artefact cannot be dismissed. Qualitatively, sodium-deficient mesophyll chloroplasts appear to possess fewer lamellae overall, although differences are not pronounced. Frequently, chloroplasts from sodium-deficient leaves contained distinct regions of parallel and unappressed stromal lamellae (u/a) (Figure 6.01e). Peripheral reticulum, i.e. a series of anastomosing tubules located in the peripheral stroma of chloroplasts and contiguous with the inner membrane of the chloroplast (Laetsch 1971), is evident in mesophyll chloroplasts of both sodium-deficient and control leaves. There does not appear to be any substantial difference in the extent of peripheral reticulum development between sodium-deficient and control chloroplasts. Although the function of the peripheral reticulum is unknown it has been implicated in the transport of metabolites (Laetsch 1971, Gracen *et al.* 1972, Chapman *et al.* 1975).

Numerous mitochondria and chloroplasts are present in the bundle sheath cells of sodium-deficient and control leaves of *C. barbata* (Figure 6.01 c, f). In sodium-deficient cells there are generally fewer organelles, although quantitative comparisons with controls were not made. Well developed grana are evident within the bundle sheath chloroplasts of both sodium-deficient and control leaves. As observed in the mesophyll chloroplasts of *C. barbata*, the bundle sheath chloroplasts of sodium-deficient leaves appear to have fewer lamellae, and also fewer grana per chloroplast, with distinct regions of parallel unappressed stromal lamellae.

Ultrastructural comparison of mesophyll chloroplasts and bundle sheath cells of sodium-deficient and control leaves of *E. indica* (Figure 6.02 b, e and 6.02 c, f) would suggest differences analagous to those observed in *C. barbata*. However, fixation of bundle sheath cells of *E. indica* was not good.

Experimental results presented in previous chapters have shown that sodium-deficient plants retain all of the fundamental characteristics of C_4 photosynthetic carbon metabolism. Anatomical and ultrastructural features of sodium-deficient leaves are in accord with this conclusion. While there would appear to be a marginal reduction in the total amount of thylakoid membranes per chloroplast, there do not appear to be any gross abnormalities in the structure of photosynthetic organelles from sodium-deficient plants.

6.3.2 Chlorophyll *a* fluorescence transients

Time courses of chlorophyll *a* fluorescence over either 1 sec, or 50 sec intervals are shown for sodium-deficient and control leaves of *C. barbata*, *E. indica* and *E. crus-galli* in Figure 6.03. The transient

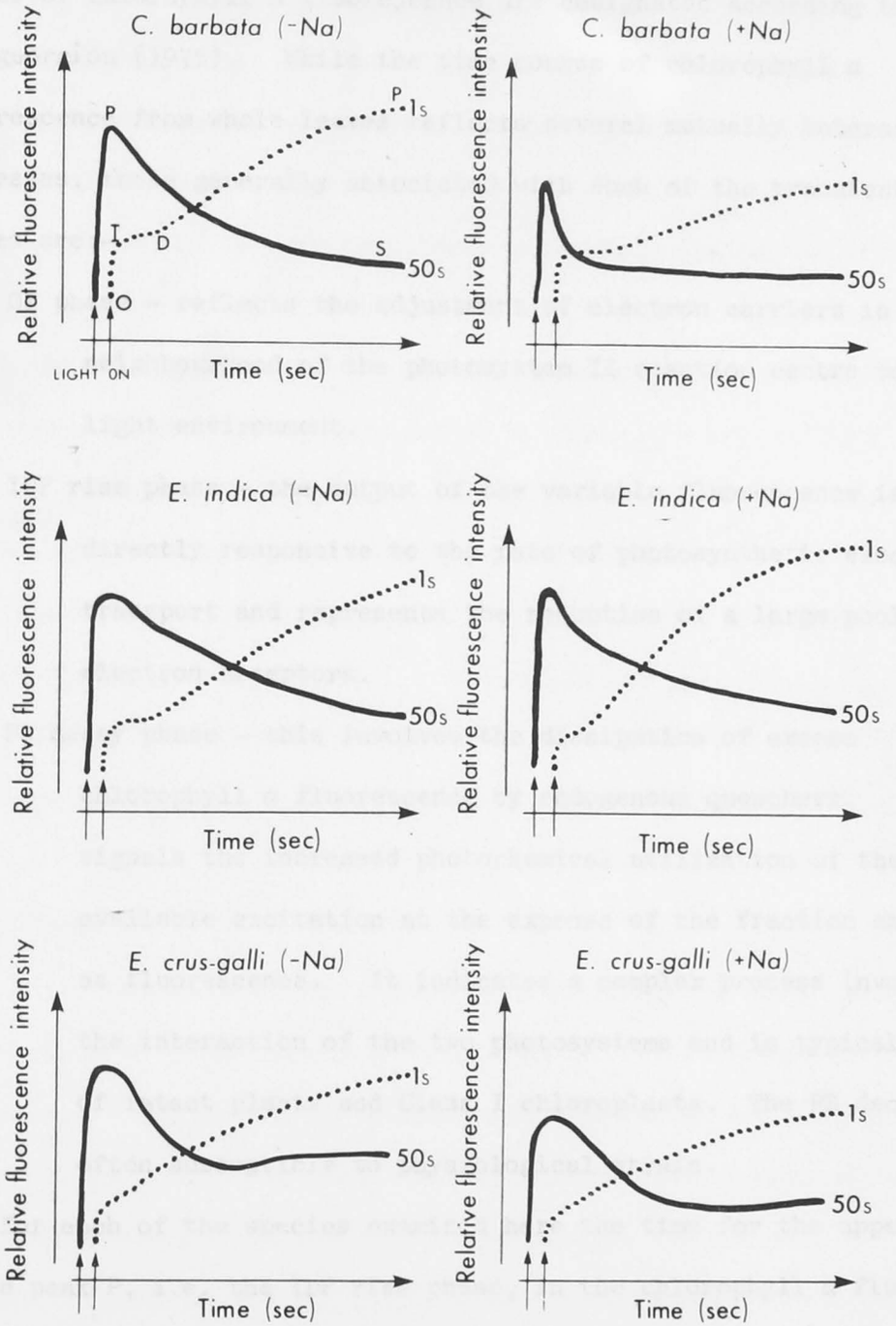


Figure 6.03 Time course of chlorophyll *a* fluorescence on two time scales (50 secs and 1 sec full scale) for sodium-deficient (-Na) and control leaves (+Na) of *C. barbata*, *E. indica* and *E. crus-galli*.

points of chlorophyll *a* fluorescence are designated according to Papageorgiou (1975). While the time course of chlorophyll *a* fluorescence from whole leaves reflects several mutually interactive processes, those generally associated with each of the transient phases are:-

OI phase - reflects the adjustment of electron carriers in the neighbourhood of the photosystem II reaction centre to the light environment.

IDP rise phase - the output of the variable fluorescence is directly responsive to the rate of photosynthetic electron transport and represents the reduction of a large pool of electron acceptors.

PS decay phase - this involves the dissipation of excess chlorophyll *a* fluorescence by endogenous quenchers. It signals the increased photochemical utilization of the available excitation at the expense of the fraction emitted as fluorescence. It indicates a complex process involving the interaction of the two photosystems and is typical only of intact plants and Class I chloroplasts. The PS decay is often susceptible to physiological strain.

For each of the species examined here the time for the appearance of the peak P, i.e. the IDP rise phase, in the chlorophyll *a* fluorescent transients from sodium-deficient and control leaves were similar. As this measurement has been shown to be a semiquantitative monitor of photosynthetic unit size (Fork and Govindjee 1980), i.e. the number of bulk chlorophyll molecules to the number of Q (the electron acceptor of photosystem II), these results show that sodium deficiency does not alter the stoichiometry of chlorophyll:electron acceptor (Q) at PSII. In sodium-deficient leaves of *C. barbata* and *E. indica* the PS decay

phase is slower than that of the control leaves. While this difference may reflect depressed photochemical utilization by subsequent processes, e.g. light activation of pyruvate, Pi dikinase, photosynthetic CO_2 assimilation, etc., these suggestions are wholly speculative. The chlorophyll *a* fluorescent transients taken from the leaves of C_4 plants should be interpreted with caution. Quite apart from problems of differential light scattering and attenuation related to the physical properties of leaves, the possession of dimorphic chloroplasts, in NADP-ME-types especially, could influence the fluorescent transient depending upon population of chloroplasts irradiated.

Essentially the characteristics of chlorophyll *a* fluorescence in sodium-deficient and control leaves are similar and do not imply considerable differences between the primary photochemical processes in these plants.

6.4 SUMMARY

While sodium-deficient leaves of both *C.barbata* and *E. indica* are thinner in cross-section, "Kranz-type" C_4 leaf anatomy is still expressed. Chloroplasts of sodium-deficient leaves are marginally smaller, appear to contain fewer lamellae and seem swollen in comparison to those of control leaves. One distinctive feature is the presence of regions of parallel and apparently unappressed stromal lamellae. However, gross abnormalities in the ultrastructure of photosynthetic organelles were not evident as a result of sodium-deficiency.

Characteristics of the IDP rise phase in chlorophyll *a* fluorescence transients did not imply marked dissimilarities in the primary photochemical processes of sodium-deficient and control leaves.

CHAPTER 7

SYNTHESIS AND SPECULATION

To search for mechanism prematurely may be following a siren's call. A good description of a phenomenon at a given level in the hierarchy not only gives a firm stepping off point for a reductionist approach, but it allows the person working at the next higher level to synthesize an understanding of his problem.

J.H.M. Thornley

Research Strategy in the Plant Sciences
Plant, Cell and Environment (1980)3, 233-236

This chapter seeks to summarize and evaluate our current understanding of the physiological consequences of sodium deficiency in C_4 plants. Firstly, experimental evidence from the preceding chapters is used to show that the C_4 pathway is operative in sodium-deficient plants, and is responsible for the initial fixation of CO_2 assimilation from the atmosphere. The characteristics of C_4 photosynthesis in sodium-deficient plants are further discussed in relation to suggested metabolic roles for sodium (Chapter 1, Section 1.3.4-5). Finally, a general account of the physiological consequences of sodium deficiency is used to highlight certain disproportionalities within the photosynthetic metabolism of these plants. These are discussed and related to proposed avenues for further research.

7.1 SYNTHESIS

Dry-weight yield responses of various species to 0.1 mol m^{-3} sodium (Section 2.3.2) demonstrated a requirement for sodium irrespective of the mechanism employed for decarboxylation of C_4 acids. These results confirm

and extend the established correlation between the essential requirement for sodium and possession of the C_4 photosynthetic pathway (Brownell and Crossland 1972). However, experiments described in the preceding chapters provide unequivocal evidence that the C_4 pathway remains functional in sodium-deficient C_4 plants. Thus, leaf anatomy and ultrastructure (Section 6.3.1), the plant carbon isotope composition (expressed as a $\delta^{13}C$ value, Section 2.3.4), the low CO_2 compensation point (Section 3.3.1) and the characteristics of radiotracer kinetics during steady-state photosynthesis (Section 4.3.1) in sodium-deficient plants effectively conform to criteria commonly used to define C_4 plants. Sodium deficiency therefore appears to diminish the overall capacity for C_4 photosynthesis without altering the basic mechanism of this process. In an attempt to determine metabolic blocks within the pathway, and thus specific sites where sodium may play its essential role(s), C_4 photosynthesis was characterized in sodium-deficient plants.

In Chapter 1 (Sections 1.3.1-5) a number of possible metabolic roles were discussed in relation to current interpretation of the C_4 pathway. In particular, two of these hypotheses were tested experimentally in the course of this study and are now re-assessed.

In recognising that certain enzymes have a common and critical role in all C_4 species, it was suggested that sodium might be required for either formation, or activation, of those enzymes involved in the conversion of pyruvate to phosphoenolpyruvate during C_4 photosynthesis (Section 1.3.4). These enzymes are: pyruvate, P_i dikinase, adenylate kinase and pyrophosphatase. The *in vitro* activities of pyruvate, P_i dikinase obtained from sodium-deficient leaves were clearly sufficient to account for the observed rates of photosynthetic CO_2 assimilation measured in these plants (Section 5.3.1, Table 5.01). Moreover, the high activities of this

enzyme obtained immediately after extraction from pre-illuminated leaves suggested that sodium deficiency did not prevent the light activation of pyruvate, P_i dikinase *in vivo* (Table 5.01). Experiments with isolated mesophyll chloroplasts from sodium-deficient leaves of *D. sanguinalis* further demonstrated that the processes associated with the regeneration of PEP from pyruvate are not directly affected by sodium nutrition (Section 5.3.2, Table 5.03). No precautions were taken to achieve sodium-free extraction or assay conditions in these experiments. However it can be stated that if sodium is directly involved in the activation of these enzymes, it would have to be effective at sodium concentrations lower than 0.2 mM (Section 1.3.5).

The kinetics of [^{14}C]-labelling during steady-state photosynthesis in sodium-deficient leaves did not provide conclusive evidence of a single metabolic block in the conversion of pyruvate to PEP. Sodium-deficient leaves of *K. childsii* (an NADP-ME C_4 plant) were characterised by increased [^{14}C]-labelling of alanine in both time-course and pulse-chase experiments (Section 4.3.1, Figure 4.03). While this might indicate an accumulation of pyruvate in the mesophyll cells during steady-state photosynthesis (ie. conversion of pyruvate to alanine *via* alanine aminotransferase), similar increases in [^{14}C]-labelling of alanine were not seen in radiotracer kinetic experiments with sodium-deficient leaves of another C_4 species (Section 4.3.1, Figure 4.04). Furthermore, comparisons of the total free amino acid pool sizes in the leaves of sodium-deficient and control plants of several C_4 species, showed that the total free amino acid concentration of sodium-deficient leaves were substantially higher than those of control plants (Section 4.3.2). The amino acids making the major contribution to these differences were; alanine, glycine, glutamic acid, serine and aspartic acid (Section 4.3.2, Tables 4.01, 4.02). The fact that the amount of [^{14}C]-label appearing in photosynthetic

intermediates is partly a function of their pool size, and that the total alanine pool of sodium-deficient leaves of *K. childsii* was very much higher than that of control leaves, suggests that the accumulation of label in alanine does not necessarily reflect a metabolic block in the conversion of pyruvate to PEP during steady-state photosynthesis in sodium-deficient leaves of this species. While the overall accumulation of alanine, and other free amino acids, in sodium-deficient leaves of C_4 plants may intimate specific disfunctions in the photosynthetic carbon metabolism of these plants, simple analysis of total leaf pool sizes could not qualify this suggestion (Section 4.3.2).

On the basis of the above observations, I conclude that sodium is not directly involved in the formation or activation of enzymes responsible for the conversion of pyruvate to PEP during C_4 photosynthesis.

A second hypothesis examined in this study proposed that the bundle-sheath compartment of sodium-deficient C_4 plants is "leakier" (Chapter 1, Section 1.3.5). This could allow increased back-diffusion of CO_2 produced in the bundle sheath during the decarboxylation phase of the C_4 pathway. The re-fixation of this CO_2 *via* PEP carboxylase would therefore diminish the overall photosynthetic efficiency of sodium-deficient C_4 plants. As the quantum yield for photosynthetic CO_2 assimilation is unaltered in sodium-deficient leaves (Section 3.3.3, Figure 3.04), and increased back-diffusion of CO_2 would be expected to increase the intrinsic energy requirements for C_4 photosynthesis (and decrease the quantum yield) it seems unlikely that this process occurs in sodium-deficient plants. Moreover, theoretical estimates of back-diffusion in sodium-deficient and control plants, suggested that in sodium-deficient plants a smaller proportion of CO_2 produced by decarboxylation leaks out of the bundle-sheath compartment (Section 3.3.4, Table 3.04).

However, as light-saturated rates of photosynthesis in sodium-deficient plants are unaffected by changing oxygen concentrations (Section 3.3.6, Table 3.05, Figure 3.10) and observed rates of O_2 uptake during steady-state photosynthesis are low and largely insensitive to changing atmospheric CO_2 concentrations (Section 3.3.7, Figure 3.11), it would seem that the CO_2 concentration in the bundle sheath of sodium-deficient C_4 plants is sufficient to effectively abolish the direct fixation of atmospheric oxygen by RuP_2 carboxylase-oxygenase. Clearly, precise estimates of the CO_2 concentration within the bundle sheath of sodium-deficient leaves cannot be made from these experimental observations. Consequently, the relatively higher rates of photosynthetic oxygen uptake (Section 3.3.7) and the enhanced [^{14}C]-labelling of glycine and serine (Section 4.3.1, Figure 4.04) observed in sodium-deficient leaves of *C. barbata* might reflect a reduced bundle sheath [CO_2/O_2] ratio during steady-state C_4 photosynthesis in these plants. Importantly, the quantum yields of sodium-deficient plants do not suggest that there is a reduction of photosynthetic efficiency as a consequence of increased photorespiration in these plants. However, the possibility exists that the combination of reduced back-diffusion of CO_2, together with increased photorespiration in sodium-deficient plants, may result in no nett change in quantum yield.

While recent experiments of Brownell (unpublished work), showing that sodium-deficiency can be either exacerbated by growing plants under low ambient CO_2 concentrations (100 µbar CO_2) or partly alleviated by growth under very high ambient CO_2 concentrations (1500 µbar CO_2) might support the notion that sodium-deficient plants are "leakier", the experimental results presented in this study do not support this hypothesis.

7.2 SPECULATION

I conclude that observed characteristics of C_4 photosynthesis in sodium-deficient plants do not wholly support any of the previously suggested metabolic roles for this element in C_4 plants (Section 1.3.1-5). Instead, experimental observations suggest that disproportionate activities of carbon metabolism and energy transduction on the one hand, and mesophyll and bundle sheath metabolism on the other, might underlie C_4 photosynthesis in sodium-deficient plants. Evidence supporting this interpretation, at different levels of the functional hierarchy in C_4 plants, is presented.

A fundamental characteristic of photosynthetic CO_2 assimilation in sodium-deficient C_4 plants is that the intercellular $p(CO_2)$, obtained at ambient CO_2 concentrations, is consistently higher than that of control leaves (Section 3.3.4). This characteristic persists irrespective of either C_4 species, or growth regime (eg. Figure 3.07(a) and Figure 7.01), and is strong evidence that stomatal conductance is not limiting photosynthetic capacity in these plants (Section 3.3.5). Furthermore, it distinguishes sodium-deficiency from other mineral deficiencies in C_4 plants (Wong 1979a,b, Wong *et al* 1979). For example, in *Z. mays* deficiency of either nitrogen, or phosphorous, results in the co-ordinated decline in both photosynthetic capacity and stomatal conductance such that intercellular $p(CO_2)$ remains approximately constant. Wong *et al* (1979) speculate that this frequently observed correlation, between stomatal conductance and photosynthetic capacity, reflects a direct response by stomata to photosynthetic metabolites (such as ATP, NADPH and ribulose bisphosphate). While the nature of the relationship between stomatal physiology and photosynthetic capacity has not been resolved, it is significant that it has been perturbed through sodium-deficiency.

Furthermore, earlier saturation of the responses of CO_2 assimilation to both irradiance and intercellular $p(CO_2)$ further characterises C_4

photosynthesis in sodium-deficient plants (Section 3.3.2-4). This suggests that reactions which limit photosynthetic capacity in sodium-deficient plants, while not apparent under low intercellular $p(CO_2)$ or low irradiance, are exacerbated as photosynthetic CO_2 assimilation increases with either increasing irradiance, or increasing intercellular $p(CO_2)$. Moreover, tentative comparisons of *in vitro* activities of both PEP carboxylase, and RuP_2 carboxylase, and the observed rates of photosynthetic CO_2 assimilation in sodium-deficient leaves would suggest that the carboxylation capacity, as such, does not limit the overall photosynthetic capacity of these plants (Section 3.3.4, Table 3.03). Thus kinetic characteristics of both CO_2 and light response curves, together with this apparent disparity between potential and observed photosynthetic rates in sodium-deficient leaves, may reflect limitations associated with the regenerative phases within both bundle sheath (ie. RuP_2 regeneration) and mesophyll compartments (ie. PEP regeneration) during photosynthetic CO_2 assimilation. In effect, I suggest that a disproportionality exists between carbon metabolism and energy transduction (ie. the generation of ATP and NADPH by light dependent processes within chloroplast membranes) and results in reduced capacity for PEP regeneration on the one hand, and RuP_2 regeneration on the other: thereby limiting the rates of photosynthesis in sodium-deficient plants.

Comparisons of electron transport capacities in isolated bundle sheath and mesophyll chloroplasts, from sodium-deficient and control C_4 plants, were not made in this study. However, increased (^{14}C)-labelling of glycine and serine and observed changes in the relative labelling of malate and aspartate, during steady-state photosynthesis in sodium-deficient leaves of *C. barbata* (Section 4.3.1, Figure 4.04) could reflect a decreased capacity for photophosphorylation in this plant. Thus, enhanced labelling of glycine and serine could suggest

a limitation in the conversion of serine through to 3-PGA within the photorespiratory carbon oxidation pathway (ie. ATP limitation of reaction catalysed by glycerate kinase), while reduced labelling of aspartate could suggest a limitation in the overall decarboxylation of aspartate *via* PEP carboxykinase (ie. a decarboxylase enzyme requiring ATP). Moreover, the diversion of photosynthate to alanine, glycine and serine, as evidenced by the increased pool sizes of these metabolites in sodium-deficient leaves (Section 4.3.2), might further reflect longer term limitations in the conversion of pyruvate to PEP, or glycerate to 3-PGA. However, the characteristics of 3-PGA dependent O_2 evolution by isolated mesophyll chloroplasts from sodium-deficient and control leaves of *D. sanguinalis* were similar (Section 5.3.2, Figure 5.02). This observation does suggest that the relationship between carbon metabolism and energy transduction are comparable in these organelles.

Perhaps the component reactions of the C_4 pathway within bundle sheath and mesophyll cells are functional, but not well co-ordinated throughout steady-state C_4 photosynthesis in sodium-deficient plants. Indeed, there is evidence which suggests disproportionality between bundle sheath and mesophyll metabolism.

For example, relative activities of PEP carboxylase (localised in the mesophyll) and RuP_2 carboxylase (localised in the bundle sheath) in the leaves of sodium-deficient C_4 plants are substantially different from those of control plants (Table 3.03, Table 5.01). Furthermore, it would seem that while the enzyme complements of bundle sheath and mesophyll cells of sodium-deficient and control plants are comparable, the ratios of bundle sheath to mesophyll enzymes are different in sodium-deficient leaves. These are given in Table 7.01. These ratios were calculated from the enzyme activities obtained in whole leaf extracts (Section 5.3.1, Table 5.01) and consider only those C_4 enzymes known

Table 7.01 Tentative estimates of the enzyme complements of mesophyll and bundle sheath cells of sodium-deficient and control plants based on enzyme activities shown in Table 5.01.

SPECIES AND TREATMENT		Mesophyll ($\frac{\text{PEP carboxylase}}{\text{Pyruvate, Pi dikinase}}$)	Bundle sheath ($\frac{\text{Decarboxylase}}{\text{RuP}_2\text{ carboxylase}}$)	Mesophyll:bundle sheath ($\frac{\text{PEP carboxylase}}{\text{RuP}_2\text{ carboxylase}}$)
E. crus-galli	+Na	7.2	1.5	4.0
	-Na	5.9	1.0	7.3
C. barbata	+Na	15.4	0.9	8.1
	-Na	10.7	0.8	16.8
E. indica	+Na	5.5	-	4.7
	-Na	4.1	-	7.3

to be strictly compartmented either within bundle sheath, or mesophyll cell type. An implicit assumption in such comparisons is that the distribution of total leaf chlorophyll between bundle sheath and mesophyll compartments is not altered through sodium-deficiency. Tentative support for this assumption is that the total leaf chlorophyll a/b ratios of sodium-deficient and control NADP-ME type C_4 plants are similar (Section 2.3.2, Table 2.03).

From Table 7.01, it is evident that while the ratios of those enzymes located in either bundle sheath or mesophyll cells are similar in sodium-deficient and control plants, overall comparison of the mesophyll and bundle sheath enzymes suggest disproportionately higher mesophyll activities in sodium-deficient leaves. At this time a distinction between chloroplastic and non-chloroplastic enzymes is not made.

In recognising that C_4 photosynthesis involves "an intricate complex of co-operative processes, based on compartmentation and transport" (Hatch and Osmond 1976), it is most likely that a disfunction in the co-ordinated expression of the mesophyll and bundle sheath genome, as a consequence of sodium-deficiency, would seriously impair the photosynthetic competence of C_4 plants. While the dimorphic chloroplasts of C_4 plants are known to contain identical genomes (Walbot 1977), and there is some evidence to suggest qualitative changes in the component processes of the C_4 pathway during leaf ontogeny (Perchorowicz and Gibbs 1980), there is only a meagre understanding of the processes involved during development of the C_4 syndrome, at the present time.

On the basis of the above observations, I suggest that a detailed examination of the development of C_4 photosynthesis in sodium-deficient plants would greatly enhance our understanding of the metabolic role of this element in C_4 plants.

7.3 SUGGESTED AREAS FOR FURTHER RESEARCH

When concluding a review of the central role of phosphoenolpyruvate in plant metabolism, Davies (1979) commented that "... there are no clear conclusions, no broad generalizations; instead there are problems galore, loose ends to be taken up, and central problems to be attacked." These are exactly my sentiments with respect to sodium-deficiency in C_4 plants.

Some of the central problems needing further research would seem to include:-

(*a*) Recovery experiment.

In a preliminary experiment gas exchange characteristics of sodium-deficient plants of *A. edulis* and *C. barbata* were monitored following supply of sodium to the culture solution. Figure 7.01. shows responses of photosynthetic CO_2 assimilation to intercellular $p(CO_2)$ for sodium-deficient and control plants, and for plants recovering from

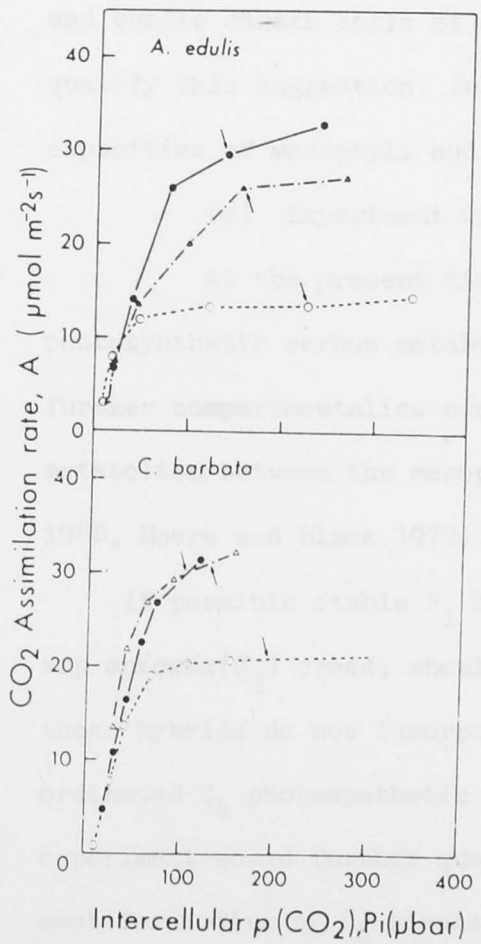


Figure 7.01:

CO_2 response curves of sodium-deficient (○), control (●) and recovering (△) (receiving 0.1mol m^{-3} sodium 62 hrs prior to gas exchange measurements). plants of *A. edulis* and *C. barbata*.

Leaf temperature was 28 C, vapour pressure difference was 20 m bar, and irradiance was 1.4 $mEm^{-2}s^{-1}$.

Values of A and P_i corresponding to P_a = 330μbar are indicated by (▲). For *C. barbata* these were obtained by interpolation of (P_a vs P_i).

sodium deficiency. In these experiments sodium was added approximately 62 hrs. prior to measuring the CO_2 response curves of the recovering plant. Clearly, recovery is rapid, as intercellular $p(CO_2)$, (obtained at ambient CO_2 concentrations) and the responses of CO_2 assimilation to high intercellular $p(CO_2)$ in the recovering plants are similar to those of controls.

These observations do not describe an early response to sodium. However, they suggest that changes in the intercellular $p(CO_2)$ of sodium-deficient plants immediately after supplying sodium, might contribute toward an understanding of mechanisms involved in recovery eg.activation of enzymes, synthesis of proteins.

(*b*) Intercellular distribution of enzyme activities.

Results obtained in this study suggest a disproportionality in the activities of certain enzymes in mesophyll and bundle sheath metabolism of sodium-deficient C_4 plants. Comparisons of the activities of chloroplastic and non-chloroplastic enzymes in isolated mesophyll and bundle sheath cells of sodium-deficient and control plants would qualify this suggestion. In addition comparisons of the electron transport capacities of mesophyll and bundle sheath compartments should be made.

(*c*) Experiment with C_4 X C_3 *Atiplex* hybrids

At the present time sodium is thought to be involved in C_4 photosynthetic carbon metabolism. However it is now clear that C_4 plants further compartmentalise component reactions of both nitrogen and sulphur metabolism between the mesophyll and bundle sheath (Gerwick *et al* 1980, Moore and Black 1979).

If possible stable F_1 hybrids from an *A. rosea*(C_4) X *A. patula* ssp *spicata*(C_3) cross, should be examined for a sodium requirement. As these hybrids do not incorporate significant amounts of carbon via a co-ordinated C_4 photosynthetic pathway (Hatch et al 1971), the outcome of this experiment would further qualify our current understanding of the requirement for sodium by C_4 plants.

(*d*) Sodium activation of key C_4 enzymes

At the present time assays of key C_4 enzymes have only been carried out in assay media with 0.2mM sodium as an impurity. No activation has been observed. However purification of components of the assay medium, together with dialysis of enzymes, should be used to further reduce the levels of sodium.

(*e*) Co-ordinated examination of the development of chlorophyll and C_4 enzymes during re-greening of etiolated seedlings of *E.crus-galli* germinated with or without added sodium.

APPENDICES

A.1 DESCRIPTION OF THE CLOSED GAS EXCHANGE SYSTEM

The closed gas exchange system used for the determination of CO_2 compensation point was that of Boag and Brownell (1979). A brief description of this system, subsequently modified to allow the measurement of oxygen exchange in intact leaves (Canvin *et al.* 1980), is given below.

Intact leaves were enclosed in a leaf chamber, specifically designed to accommodate thin leaf blades, and then allowed to equilibrate in air at 2 mE m^{-2} s^{-1} (400-700 nm). Illumination was from a 1000 W Hg-vapour lamp. After equilibration the system was flushed with argon and the required amount of $^{18}O_2$ (99% ^{18}O Norsk Hydro, Oslo, Norway) was injected into the system via a two-way valve. The system was then closed and the gas was circulated by a metal bellows pump. Mass 32, Mass 36 and Mass 40 were continuously monitored with a GD 150/4 mass spectrometer.

The CO_2 concentration was controlled by varying the pressure on a calibrated capillary which bled pure CO_2 into the system. The CO_2 concentration was measured with an IRGA analyser (UNOR-2, Maihak, Hamburg, Germany) and CO_2 uptake, at constant CO_2 concentration in the system, was calculated from the rate of CO_2 addition.

Rates of O_2 uptake and O_2 evolution were calculated according to

Canvin *et al.* (1980).

Each measurement was averaged over a 10-12 minute period of gas exchange after steady-state conditions were obtained. As the total gas pressure increased, due to O_2 production, the system was equilibrated to atmospheric pressure between measurements. Water vapour in the system was collected in an ice trap.

Leaf temperature was measured with a copper-constantan thermocouple (42 S.W.G., 0.1 mm diameter), appressed to the undersurface of the leaf, and was maintained at 28°C.

At the conclusion of each experiment leaf material was excised and estimates of total leaf area and chlorophyll were made.

A.2 OPEN GAS EXCHANGE SYSTEMS

In this study rates of transpiration and assimilation of CO_2 were made in two open gas exchange systems: designated open systems (I) and (II). A brief description of each system is given below.

A.2.1 Open system (I)

This gas exchange system, essentially that of Wong (1979b), was used for experiments with dicotyledonous species and in quantum yield determinations.

Gas exchange measurements were made using a small (2 x 1.2 ml) double-sided glass and aluminium leaf chamber clamped onto the leaf. Illumination was provided by a water-cooled xenon arc lamp (Osram.XBF, 2500 W), the UV and IR components were removed with a Schott KG2B filter. Irradiance could be varied by interposing copper screens having various mesh widths and, in the case of quantum yield estimates, by using calibrated neutral density filters (Balzers, Leichenstein).

Quantum flux density (400-700 nm) was measured with a quantum sensor (Model LI-190 SR, Lambda Instruments, Lincoln, Nebraska) which was placed in the leaf chamber prior to the commencement of experiments. Irradiance could not be measured while leaf material was within the chamber. Leaf temperature was monitored with a copper-constantan thermocouple (42 S.W.G., 0.1 mm diameter) appressed to the under-surface of the leaf, and was maintained at 28°C.

Air was passed through each side of the chamber at a rate of 0.256 mmol s^{-1} (400 ml min^{-1}). The CO_2-free air, obtained by passing compressed laboratory air through two columns of soda-lime in series (Carbosorb, BDH Chemicals Ltd.), was first humidified and then passed through a condensor maintained at 18°C. CO_2 was added by a Hastings flow meter connected to a Hastings flow controller. The relative humidity of the air entering each side of the chamber was measured with two Humidicap humidity sensors (Vaisala Co., Finland) maintained at constant temperature. Relative humidity of the outgoing air was measured with the same sensors and readings of relative humidity converted to vapour pressure. The CO_2 concentration of the ingoing air was measured with a Beckman IRGA (Model 315B) after the air had passed through an ice-trap. The differences in CO_2 concentration between ingoing and outgoing air were measured with two Beckman IRGA's (Model 865) for upper, and lower chambers respectively. These IRGA's had been fitted with optical water filters.

The outputs of all sensors were continuously registered on a Rikadenki six-pen potentiometric recorder. However, in experiments to determine quantum yield the outputs were directly connected to an IS2-11 Digital Computer (Digital Equipment, Australia).

In this study the rates of CO_2 assimilation and transpiration are

presented as the sums of adaxial and abaxial surfaces while leaf area relates to one surface only.

A.2.2 Open system (II)

This gas exchange, a modification of that described by Powles and Osmond (1978), was used for experiments with monocotyledonous species.

Gas exchange measurements were made using a glass and aluminium leaf chamber specifically designed to accommodate thin leaf blades (total volume 150 mls). A small horizontal fan circulates the air past the leaf and provides rapid mixing within the chamber so as to ensure that the composition of the air is virtually uniform. Illumination was provided by a water-cooled xenon arc lamp (Osram XBF, 2500 W), the UV and IR components were removed with a Schott KG2B filter. Irradiance was varied by interposing copper screens having various mesh widths and quantum flux density (400-700 nm) was measured with a quantum sensor (Model LI-190 SR, Lambda Instruments, Lincoln, Nebraska). Leaf temperature was monitored with a copper-constantan thermocouple (42 S.W.G., 0.1 mm diameter), appressed to the undersurface of the leaf, and was maintained at 28°C.

Routinely, CO_2-free air was obtained by passing compressed laboratory air through two columns of soda-lime in series. In those experiments where ambient oxygen concentration was varied the CO_2-free air stream was mixed with either pure N_2 or pure O_2. Air with the desired partial pressure of CO_2 was obtained by bleeding pure CO_2 gas through a calibrated needle valve into the CO_2-free air stream. The gas stream was humidified and then passed through a water-jacketed condensor held at the desired dew point. The ambient partial pressure of CO_2 entering the chamber and the differential between air entering

and leaving the chamber were measured with a single Beckman IRGA (Model 865). The vapour pressures in the air entering and leaving the chamber were measured with a single dew point hygrometer (Cambridge Model 880, Massachusetts, U.S.A.). A series of solenoid valves (Herion Stuttgart, F.D.R.) were used to divert gas streams before or after the leaf chamber to the IRGA and dew point hygrometer. Flow-meters, with needle valves (Flowbits, Bassingstoke, U.K.), were used to distribute gas flow throughout the system and air was passed through the leaf chamber at a rate of 1.4 mmol s^{-1} (2 litre min^{-1}) and monitored with a mass flow meter (Hastings Model EALL 5K, Virginia, U.S.A.). Outputs of all sensors were registered on a digital voltmeter (Hewlett-Packard Model 580A) and the outputs from the CO_2 analyser and humidity sensor were continuously recorded on a two-pen potentiometric recorder.

In both gas exchange systems described above the vapour pressure difference (between the intercellular spaces and ambient air) was maintained at 20 mbar.

A.2.3 The analysis of gas exchange

In gas exchange experiments described in this study the outputs of CO_2 analysers and humidity sensors were continuously monitored and allowed the calculation of rates of CO_2 and water vapour exchange, stomatal conductance to gaseous diffusion and the intercellular partial pressure of CO_2 in accordance with Wong *et al.* (1978).

The rate of transpiration per unit leaf area was determined as:

$$E = \frac{u(e_a - e_o)}{aP} \qquad (1)$$

where u is the molar flux of air through the chamber, e_a is the vapour pressure in air leaving the chamber, e_o is the vapour pressure in air entering the chamber, a is leaf area, and P is the total air pressure.

The rate of CO_2 assimilation per unit leaf area was determined as:

$$A = \frac{u(P_o - P_a)}{aP} \qquad (2)$$

where P_o and P_a are the partial pressures of CO_2 in air streams entering and leaving the chamber respectively.

Leaf conductance to vapour transfer was determined as:

$$g = \frac{EP}{e_i - e_a} \qquad (3)$$

where e_i, the vapour pressure in the intercellular spaces, is taken as the saturation vapour pressure at leaf temperature. Thus, the dimensions of g are the same as E, i.e. molar flux density.

The partial pressure of CO_2 in the intercellular gas spaces was determined as:

$$P_i = P_a - 1.6\frac{PA}{g} \qquad (4)$$

the numerical factor 1.6 being the ratio of the diffusivities of water vapour and CO_2 in air. In using this relationship it is assumed that non-stomatal conductance is a very small component of total leaf conductance to vapour transfer in the light. Typically, as in the case of control plants of *C.barbata* where stomatal conductance in the dark ($g = 0.032$ mol m^{-2} s^{-1}) is very much less than in the light ($g = 0.505$ mol m^{-2} s^{-1}), this assumption is valid. This is not the case with *A. nummularia* and estimates of g and P_i for this species should be interpreted with caution.

Calculated rates of CO_2 assimilation and leaf conductance are expressed in terms of the area of one side of the leaf.

A.3 AMINO ACID ANALYSIS

Model: Beckman Amino Acid Analyser 119CL

Single column analysis: Resin - Beckman W3

Column size - 6 x 220 mm

Column temperature - 50°C/65°C change up at 20 min

Buffers: 1st elution buffer (A) - Na citrate pH 3.25

2nd elution buffer (B) - Na citrate pH 3.95

3rd elution buffer (C) - Na citrate pH 6.40

Change over time from (A) to (B) - 24 min

Change over time from (B) to (C) - 38 min

Buffer flow rate - 44 ml/hr

Regeneration buffer: 0.2 N NaOH

Ninhydrin reagent flow rate: 22 ml/hr

Chart flow rate: 6 in/hr

Time taken for complete analysis: approximately 90 min

Samples: Standard amino acids for calibration: 25 nmoles per amino acid except for Trp (20 nmoles) and Cys (12.5 nmoles).

Sample volume - 10 or 100 µl

The analyser was coupled to a Beckman Model 126 Data System which converted the area of the amino acids eluted into absolute values (nmoles).

A.4 MANGANESE TOXICITY IN Japanese Millet

In preliminary growth experiments, both control and sodium-deficient plants of Japanese millet *(E. crus-gulli/E. utilis)* grew poorly in the basal culture solution (see Section 2.2.4). Depressed yields were associated with pronounced interveinal chlorosis and the presence of brown necrotic lesions on older leaves. While these symptoms resembled those of iron deficiency, the addition of higher concentrations of iron to the nutrient solution did not alleviate leaf chlorosis. However, it became evident that these symptoms were less pronounced when cultures contained eight plants, rather than four, per 2-litre nutrient solution. This observation suggested that symptoms were those of manganese toxicity, as manganese toxicity can resemble iron deficiency in certain species (for review see Foy *et al* 1978).

Subsequently, control plants (+Na) of Japanese millet, comprising of either four or eight plants per 2-litre culture vessel, were grown in the basal culture solution with varying concentrations of

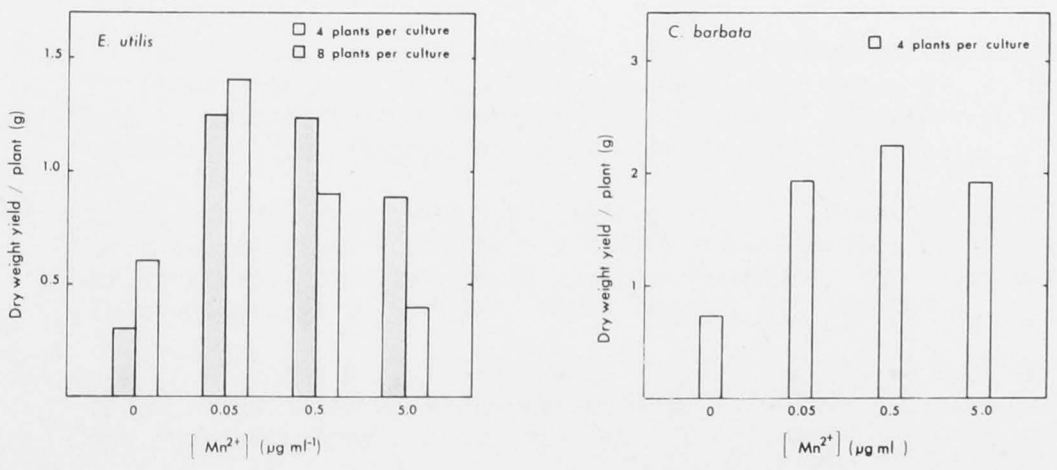


Figure A.4.1 Yield of Japanese Millet *(E. utilis)* and *C. barbata* as a function of the concentration of manganese in solution.

manganese. Visual symptoms, of either manganese toxicity or deficiency, and the total dry weight yield for each treatment were recorded (see Figure A.4.1). In addition the responses of *C. barbata* to different manganese concentrations were examined. The results of these experiments (Figure A.4.1) confirmed that the concentration of manganese in the basal culture solution (ie. 0.5 μg ml^{-1}) was toxic for Japanese millet and that this toxicity was most pronounced in those cultures which contained only four plants.

Consequently, experimental plants of Japanese millet were routinely grown in a basal culture solution containingly only 0.05 μg ml^{-1} manganese. Within the time-course of growth experiments visible symptoms of manganese deficiency were not evident in either control or sodium-deficient plants.

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